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13. ABSTRACT *(Maximum 200 words)*

Cancer cells often contain disruptions in the transition from G1 phase to S phase. The retinoblastoma protein (pRb) is one negative regulator ofcellular proliferation. Many breast cancers retain functional pRb and therefore must use other mechanisms to alleviate the tumor-suppressive function of pRb. One common mechanism to circumvent pRb function is amplification of the G1 phase cyclin dependent kinases (cdk), cdk4 and cdk6. Cdk4 and cdk6 are thought to have redundant functions as pRb regulators, despite an increasing body of evidence suggesting that certain tumor types specifically activate either cdk4 or cdk6. This implies that either the function or the regulation of these kinases may be different in different cell types. Data presented here show that cdk4 and cdk6 have different abilities to drive the cell cycle from G1 into S phase in the osteosarcoma cell line, U2OS and in primary mouse astrocytes. Understanding differential regulation of these two kinases and their potentially different activation in response to mitogenic signals may eventually provide a mechanism to therapeutically interrupt kinase activity. Ability to interrupt kinase activity separate from cyclin D1 association may prove an important intervention in breast cancer.

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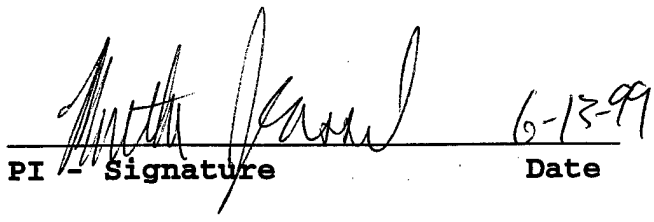

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INTRODUCTION:

Cancer is characterized in part by a loss of cellular growth control. An important regulator of cellular growth is the retinoblastoma protein (pRb) which has been found to be inactivated in several tumor types. Deregulation of the "pRb pathway" can result from mutations of both positive and negative regulators of pRb. For example, in breast cancers cyclin D1 is often amplified. Cyclin D1 activates cyclin dependent kinase 4 (cdk) and cdk6, to phosphorylate pRb and inactivate its cell cycle inhibitory function. The D-cyclin kinases, cdk4 and cdk6, are often amplified in human cancers and inhibitors of kinase function are often deleted in tumors. The purpose of the research presented here is to investigate the pathways by which cdk4 and cdk6 stimulate unregulated cell division and the mechanism by which these kinases are activated. Numerous reports have shown that increased expression and protein levels of cdk4 and cyclin D proteins are found in breast cancer. The experiments presented here are aimed at furthering our understanding of cdk4 function in cell growth regulation. Results and experiments summarized here suggest novel mechanisms of regulation of cdk4 and its homolog cdk6 and aim to investigate differential functions of cdk4 and cdk6. By understanding the roles these kinases play in proliferation, we hope to understand their function in tumors, including breast tumors.

BODY

Experiments outlined in Aim 1 and summarized in the original Statement of Work describe experiments to determine the contribution of inhibitor binding (p15, p16, p18) to kinase function as measured by pRb phosphorylation. As outlined in that Statement of Work, SAOS-2 cells were transfected with mutant forms of cdk4 and cdk6 to determine the effect of these proteins on pRb phosphorylation by immunoprecipitation. In addition, this aim described experiments designed to test the cdk6 mutants for their ability to bind cyclin D1 and the INK family of inhibitors. These experiments were scheduled to be started early in the research outline (Month 1-3 and Month 3-18, respectively).

Results of studies of pRb phosphorylation of immunoprecipitated SAOS-2 extracts revealed the following result. Transfected cdk6NFG, although determined to be kinase inactive in *in vitro* kinase assays, resulted in phosphorylation of pRb *in vivo* due to titration of p16. SAOS-2 cells express high levels of p16 and when cdk6NFG is expressed to high levels in these cells, the cdk6NFG protein binds p16, freeing endogenous cdk6(wt) from this inhibitor, presumably allowing complex formation with cyclin

D1 and resulting in pRb phosphorylation *in vivo*. In contrast, cdk6R31CNFG was determined to be unable to phosphorylate pRb *in vivo* since it has no endogenous kinase function and is unable to titrate inhibitors away from the endogenous cdk6 protein.

In addition to these studies, experiments were also undertaken to determine the ability of the cdk6 mutants to bind cyclin D1 and the INK family of inhibitors. These immunoprecipitation studies were undertaken in U2OS cells, an osteosarcoma cell line which contains detectable levels of cyclin D1 as well as the inhibitor p18, and are deleted for p16. These studies demonstrated that the R31C mutation disrupted binding of cdk6 to p18 in the context of both the single (cdk6R31C) and double (cdk6R31CNFG) mutant. Furthermore, it was found that this mutation did not disrupt the binding of these mutant proteins to cyclin D1 nor to the CIP/KIP family inhibitor p27. Concomitant with immunoprecipitation studies, cell cycle analysis of transfected U2OS cells were also undertaken. These studies resulted in a manuscript entitled "Cdk6 can shorten G1 phase dependent upon the N-terminal INK4C interaction domain", which is submitted for publication. The data in this manuscript demonstrate specific functions of cdk6 required in G1 acceleration of transfected U2OS cells, including a potential positive role for the INK family of inhibitors in cdk6 function. In addition, these studies revealed differences in the function of cdk4 and cdk6 in U2OS cells, since expression of cdk6, but not cdk4, resulted in the acceleration of G1 phase (M. Grossel, P. Hinds, unpublished observations).

The observation that cdk4 and cdk6 function differently in U2OS cells has led to further investigate functional differences between cdk4 and cdk6. However, we wish to investigations functional differences in a less perturbed, primary cell system. To perform these types of studies, we have begun collaboration with Dr. Eric Holland and Dr. Harold Varmus at the NIH. This collaboration, setup at the Colrain pRb meeting organized by Dr. David Livingston and Dr. Bob Weinburg, allows us to study the function of cdk4 and cdk6 in a primary cell line and will allow us to introduce these kinases directly into mammary cells using the same model.

The system of Holland and Varmus utilizes mice that were genetically altered to express an astrocyte-specific transgene encoding TVA, the receptor for subgroup A avian leukosis virus (ALV) (Holland and Varmus 1998) . Production of the TVA receptor by these astrocytes allows them to become infected by RCAS vectors expressing a cDNA of interest. Astrocytes are an especially interesting cell type in which to study cdk4 and cdk6 function because tumor formation suggests that cdk4, but not cdk6, plays a distinct mitogenic role in these cells. The cdk4 gene is amplified 10-100 fold in almost half of gliomas (He et al. 1994; Ichimura et al. 1996), while cdk6 amplification has not been noted in glioma, suggesting that these highly

homologous kinases have distinct roles or mechanisms of activation in some cell types. A further advantage of this system is that astrocyte infections can be performed *in vivo* by direct injection of RCAS virus and in a tissue culture system by infecting the genetically altered mouse astrocytes with RCAS virus. Finally, because the effects of cdk4 infection in GTV-a astrocytes have been well defined through the studies of Holland and colleagues (Holland et al. 1998), cdk6 function in these cells can be directly compared to previously published findings of cdk4 function. It is important to note that these GTV-a astrocytes are used as a model system for the study of kinase function in primary cells and not to study neurobiology.

Interestingly, preliminary *in vitro* studies in GTV-a astrocytes indicate that cdk4 and cdk6 indeed function differently in this model as well as in U2OS cells (M. Grossel and P. Hinds, unpublished results). In these studies, primary brain cultures from *Gtv-a* mice, in which the *tv-a* transgene is under control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter were studied. The TVA receptor allows infection by RCAS vectors encoding gag, pol and env genes of ALV, and carrying a cDNA. RCAS vectors containing the cDNA's of cdk4, cdk6 or alkaline phosphatase (AP) (as a negative control) were infected into DF-1 chicken cells to allow viral production and protein levels were analyzed by immunoblot. The supernatant of infected DF-1 cells was then applied to primary brain cultures taken from *GTV-a* transgenic mice. Since these RCAS viruses infect only cells expressing the TVA receptor, only chicken cells or genetically modified mouse cells can be infected with these viruses.

In these experiments, RCAS vectors carrying the cDNA of cdk4, cdk6 or AP were used to infect primary brain cultures isolated from newborn GTV-a transgenic mice. An unifected control was also studied for comparison. As expected, cdk4- and AP-infected astrocytes closely matched the results published by Holland et al. (Holland et al. 1998), reproducing the logrithmic growth curve of cdk4-infected astrocytes (Figure 1). However, in two separate infections, cdk6-infected astrocytes showed growth significantly slower than cdk4 and easily distinguishable from AP and uninfected control growth curves (Figure 1). The inabilty of cdk6 to cause the rapid growth observed with cdk4 expression is consisitent with tumor data indicating that cdk4, but not cdk6, is amplified in human gliomas. These data suggest that cdk4, and not cdk6, imparts a growth advantage in these cells. In addition to the differences in growth rate of cdk4 and cdk6 infected cells, morphology differences were also noted. Phase-contrast microscopy reveals striking differences in cellular morphology of cdk4- and cdk6-infected astrocytes throughout the two-month duration of the current study. As shown in Figure 2, RCAS-cdk6 infection results in cells with a spindly morphology as compared to those

infected with cdk4. Consisitent with the differences in morphology and growth rates of cdk4- and cdk6-infected cells, these two kinases were also found to localize to different cellular compartments of the infected astroyctes. Indirect immunofluorescence of cdk4-infected and cdk6-infected astrocytes show very different patterns of localization for these two highly homologous kinases that are believed to be functionally redundant. Cdk4 is seen to localize primarly to the nucleus (green staining) while cdk6 is localized primarily to the cytoplasm (green staining) (Figure 3). The differences in localization seen in these preliminary studies may explain the different effects of cdk4 and cdk6 on cell growth. Since the D-cyclin kinases function to phosphorylate pRB in the nucleus, cytoplasmic cdk6 would be unable to function in cell cycle progression and thus despiite its overexpression, the cytoplasmic kinase would not have the mitogenic effect observed with cdk4 overexpression. Thus, one cell-type specific mechanism of regulation of these kinases may be cell-type specific molecules that determine localization of cdk4 or cdk6. Regulators of cdk4/6 activity may allow the kinases access to the nucleus in the proper manner and timing for cell cycle progression. These data are consistent with our studies in U2OS cells that indicate that proper subcellular localization is critical to proper kinase function of cdk6. Future studies planned with this TV-a system include infection of TV-a expresing cells in which the *tv-a* gene is driven from the MMTV and/or WAP promoters which are breast specific. These *tv-a* chimeric mice were made by Dr. Yi Li in Dr. Harold Varmus's lab and these studies could be carried out in collaboration with Yi Li as outlined in the modified statement of work below.

In addition to the studies in the GTV-a system, I have also pursued studies of cdk4 and cdk6 function using the two-hybrid system in yeast. The two hybrid system is a nondiscriminating method to find binding partners of these kinases that may help in understanding the similarities and differences in cdk4 and cdk6 function. Proteins that bind these kinases in yeast may be substrates of the kinases or may be proteins that activate, or are activated by cdk4 and cdk6. Binding partners of cdk4/6 could also play a role in inhibiting the kinases, translocating the kinases to the nucleus, or retaining the kinases in a subcellular compartment. Becuase my hypothesis is that cdk4 and cdk6 have distinct functions, the primary goal is to identify protein partners that are specific for one, but not both, of the D-cyclin kinases. To identify differential interactors of cdk4 and cdk6, the results of two separate two-hybrid screens could be compared. Alternatively, one could perform a screen and then mate the resultant yeast with a yeast containing the alternate kinase to test binding partners of both kinases. However, rather than screen libraries twice, we have designed a novel two-

hybrid system that allows library screening with two bait proteins at the same time (M. Grossel, P. Hinds, manuscript attached).

This differential two hybrid system utilizes two distinct DNA-binding domains that drive expression of binding-domain dependent reporter genes. In our system GAL4 sites drive both HIS3 and LacZ expression while LEXA sites drive URA expression. Thus, HIS positive, blue yeast indicates and interaction with bait X (GALcdk6) while URA positive yeast indicates an interaction with bait Y (Lexcdk4). Thus a cDNA that binds cdk6 but not cdk4 would be HIS positive and blue but URA negative. The auxotrophic markers selected as reporters in these yeast allow rapid and efficient library screening. For instance HIS production can be measured by the growth of yeast in the presence of 3-aminotriazole, a drug that allows titration of histidine production. LacZ production is easily measured by a Gal-lift of colonies onto nitrocellulose, a quick freeze/thaw to lyse the yeast and incubation in X-Gal buffer. Finally, uracil production by yeast can be tracked by negative selection in the presence of Flororotic acid (FOA) which is toxic to yeast that are producing uracil.

This differential two hybrid system will be used to screen for proteins that interact with cdk6 but do not interact with cdk4, but will also identifying protein products that interact with cdk6 and cdk4 and also allows the identification of proteins that bind cdk4 better than cdk6. The goal of this project is to identify differential interactors, however, binding partners that bind to both kinases are also interesting and will saved for analysis. The two-hybrid screens will be carried out by Martha Grossel at Connecticut College and positive clones will be further analyzed *in vitro* and in mammalian cells by Kathy Marsh at Harvard Medical School, as outlined in the modified statement of work attached.

REFERENCES

He, J., J. Allen, V. Collins, R. Allalunis-Turner, R. Godbout and C. James. 1994. CDK4 amplification is an alternative mechanism to p16 gene homozygous deletion in glioma lines. *Cancer Res.* **55:** 4833-4836.

Holland, E.C., W.P. Hively, V. Gallo and H.E. Varmus. 1998. Modeling mutations in the G1 arrest pathway in human gliomas: overexpression of cdk4 but not loss of INK4a-ARF induces hyperploidy in cultured mouse astrocytes. *Genes & Dev.* **12:** 3644-3649.

Holland, E.C. and H.E. Varmus. 1998. Basic fibroblast growth factor induces cell migration and proliferation after glia-specific transfer in mice. *Proc. Natl. Acad. Sci.* **95:** 1218-1223.

Ichimura, K., E. Schmidt, H. Goike and V. Collins. 1996. Human glioblastomas with no alterations of the CDKN2A (p16INK4a, MTS1) and CDK4 genes have frequent mutations of the retinoblastoma gene. *Oncogene* **13:** 1065-1072.

Modified Statement of Work 1999-2000:

Since the Principal Investigator has taken a position at Connecticut College, the Statement of Work has been modified to split the experiments between Harvard Medical School (principal investigator, K. Marsh) and Connecticut College. Harvard Medical School will subcontract to Connecticut College.

The following will be conducted at Connecticut College by M. Grossel

Month 1-6: Screen a human cDNA library for cdk6-interacting proteins using the differential two-hybrid yeast system.
Confirm interactions in yeast, cure plasmids, sequence, match in database

Month 1-5 Infect TVA+ astrocytes with the cdk6 mutant, cdk6R31C in RCAS
Measure growth curves for 30-60 days
Perform indirect immunofluorescence to determine localization of R31C

Month 4-6: Clone cdk4 mutant, cdk4R24C into RCAS vector

Month 6-12: Infect TVA+ astrocytes with cdk4R24C
Measure growth curves for 30-60 days to determine if the cdk4 mutant can immortalize astrocytes (as cdk4 wildtype does)
Perform indirect immunofluorescence to determine localization of R24C

The following experiments will be conducted at Harvard Medical School

Month 2-12: Characterize clones identified in the two-hybrid screen
Transfect clones into mammalian cells, determine the effect of transfected clones on localization and kinase activity of cdk6.

Month 6-12: Infect TVA+ mammary epithelial cells with cdk4 and cdk6
Measure growth curves, does cdk4 immortalize in breast cells?
Perform indirect immunofluorescence to determine localization of cdk4, cdk6 and the mutants of cdk4 and cdk6

Key Research Accomplisments

- Characterization of requirements of cdk6 kinase in G1 acceleration
- Identified differences in function of cdk4 and cdk6 in U2OS cells
- Identified differences in cdk4 and cdk6 function in primary mouse astrocytes, including growth rates, morphology and cellular localization
- Developed Differential Two-Hybrid system to screen cDNA libraries with two baits at the same time.

Reportable Outcomes

Manuscripts:

1. Cdk6 can shorten G1 phase dependent upon the N-terminal INK4C interaction domain. M. Grossel, G. Baker and P. Hinds, submitted

2. A Yeast Two-Hybrid system for discerning differential interactions using multiple baits. M. Grossel, H. Wang, B. Gadea, W. Yeung, and P. Hinds, submitted.

Abstracts:

1. Keystone Cancer and the Cell Cycle Meeting.

Presentations:

1. Colrain meeting on pRb and the cell cycle.

Developments:

1. Development of the Differential Two-Hybrid system.

Employment/Funding based on training supported by this award:

1. Assistant Professorship at Connecticut College was granted to M. Grossel, where this research will continue.

2. NSF Career grant will be applied for by M. Grossel on the basis of the research and the training funded by this award.

Attachments:

Figures 1, 2, 3
cdk6 manuscript
Two-Hybrid manuscript
Keystone Meeting Abstract

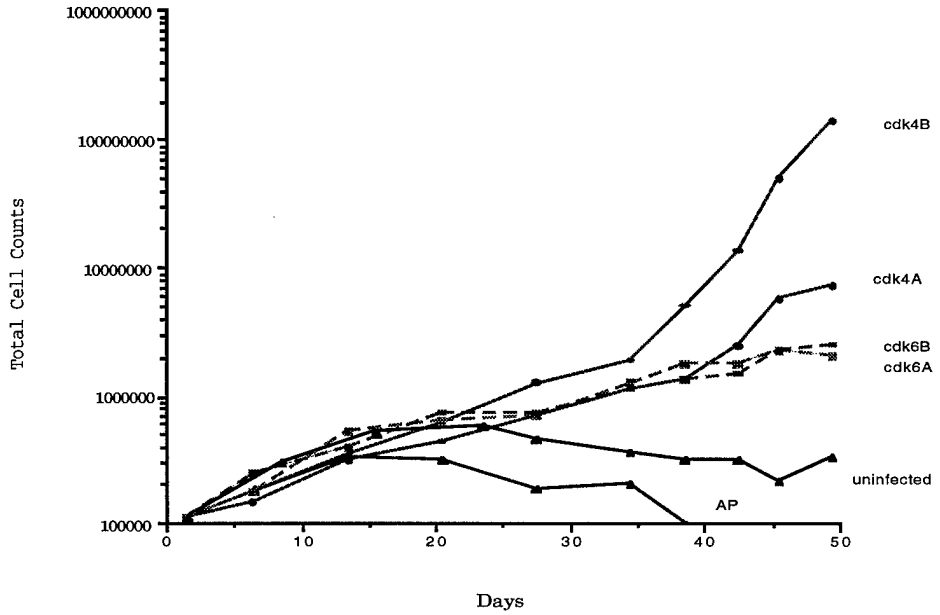
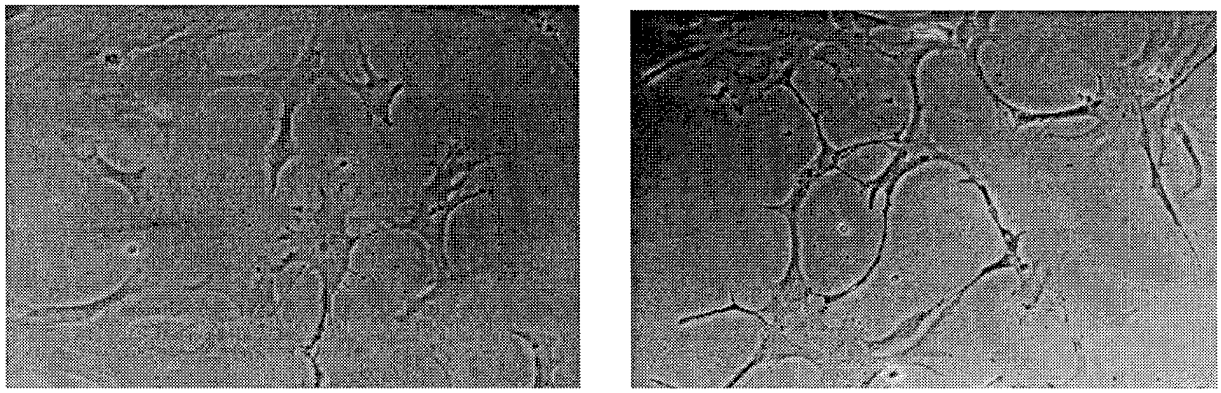
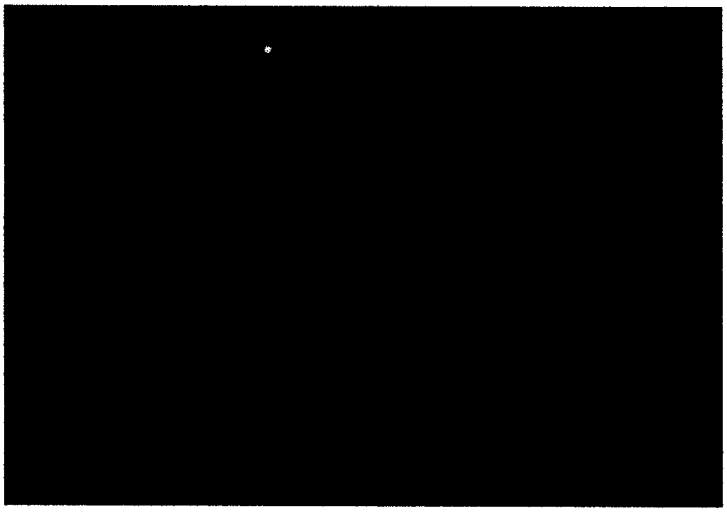


Figure 1: Growth curves comparing two separate infections of GTV-a astrocytes with RCAS-cdk4 and RCAS-cdk6 as well as RCAS-alkaline phosphatase and uninfected cells. Cells were counted and split when near-confluent over the course of 50 days.



RCAS cdk4 RCAS cdk6

Figure 2: Phase contrast images of GTV-a astroyctes infected with RCAS-cdk4 or RCAS-cdk6. The morphological differences were observed throughout the 50 day course of the experiment, these images were obtained near the end of the experimental timecourse.



RCAS-cdk4

RCAS-cdk6

Figure 3: Indirect immunofluorescence of infected GTU-a astroyctes. Red staining is anti-actin antibody and green staining is anti-kinase antibody. Cells fixed in 70% EtOH with glycine.

Cdk6 can shorten G1 phase dependent upon the N-terminal INK4C interaction domain

Martha J. Grossel, Gregory L. Baker and Philip W. Hinds*

Running title: cdk6 acceleration of G1 phase

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Summary

Deregulated activity of cdk4 or cdk6 can lead to inappropriate cellular proliferation and tumorigenesis accompanied by unchecked inactivation of the retinoblastoma tumor suppressor protein. Certain tumor types preferentially activate either cdk4 or cdk6, suggesting that these kinases may not be equivalently oncogenic in all cell types. While it is clear that cdk4 can act as an oncogene at least in part by evading inhibition by $p16^{INK4a}$, the role of cdk6 in tumorigenesis is less well understood. To investigate the function of cdk6 as an oncogene, the requirements for proliferation caused by cdk6 overexpression were studied. Cdk6-transfected U2OS cells displayed an accelerated progression through G1 phase that was dependent on kinase activity and that did not correlate with p27 binding. Further, a mutation that prevents cdk6 interaction with INK4 proteins (cdk6R31C) was found to inactivate the proliferative effect of cdk6 and increase cytoplasmic localization, despite the fact that this mutant could phosphorylate pRb *in vitro*. Together, these data suggest a role for the cdk6 INK4 interaction domain in the generation of functional, nuclear cdk6 complexes and demonstrate the importance of elevated cdk6 kinase activity in G1 acceleration.

Introduction

In mammalian cells the regulation of cell division is tightly controlled through a series of checkpoints within the cell cycle including the restriction point in late G1 phase, a checkpoint that determines commitment to DNA replication. The restriction point may be viewed as the culmination of activation of G1 cyclin-dependent kinases, enzymes that govern cell cycle progression through phosphorylation of key regulatory substrates. Specifically, the cyclin D proteins and their associated kinases, cyclin dependent kinase (cdk) 4 and cdk6, function

early in G1 phase of the cell cycle to link growth regulatory signals to the control of cell division. Both cdk4 and cdk6 can be activated by all three D-type cyclins (cyclins D1, D2 and D3) and are thought to function as positive effectors of G1 progression [1-4]. Activation of cdk4 and cdk6 allows progression from G1 phase to the start of DNA synthesis in normal eukaryotic cells by phosphorylating and inactivating the retinoblastoma protein (pRb). This initial modification of pRb by cdk4/cdk6-dependent phosphorylation may be followed by further phosphorylation by cyclin E/cdk2 complexes and ultimately relieves repression of E2F-dependent promoters, allowing the transcription of S-phase genes and the onset of DNA replication (for review see [5]).

Because the cyclin D-dependent kinases play a pivotal role linking growth regulatory signals to cell division, activity of these kinases is very tightly controlled. Kinase activity is regulated by the periodic synthesis and destruction of the cyclin subunits, by phosphorylation and dephosphorylation of the kinase subunit, and through complex formation with two families of cyclin-dependent kinase inhibitors (CKIs) [6]. The CIP/KIP family of inhibitors includes p21, p27 and p57, which associate with several different cyclin/cdk complexes [7-11]. These proteins may act as stimulators of cdk activity as well as inhibitors since p21 has been shown to both activate and inactivate cyclin/cdk complexes, perhaps dependent on stoichiometry [12, 13]. Indeed, the ability of p21 and p27 to stabilize D cyclin/cdk4(6) complexes may be required for the proper formation of these complexes [14]. In contrast to the CIP/KIP family, the INK family of CKIs ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) specifically inhibit the activities of cdk4 and cdk6 by binding directly to the kinase subunit, disallowing association with the activating cyclin D subunit [15, 16](for review see [17]).

Aberrant cell proliferation and tumorigenesis can result from deregulated activity of cdk4 and/or cdk6 with subsequent, inappropriate inactivation of pRb

in several tissue types. This increased kinase activity can result from overexpression of the regulatory subunit, cyclin D1, and also from amplification of the kinase-encoding gene. In addition, deletion or inactivation of the gene encoding $p16^{INK4a}$ frequently leads to dysregulated cdk4/cdk6 activity in human tumors, as do mutations in cdk4 that prevent its association with $p16^{INK4a}$ [18-25]. In most cases, tumors containing hyperactivated cdk4 or cdk6 retain intact *RB* alleles, suggesting that such kinase activations render pRb unable to control proliferation. These findings indicate that deregulated cdk4 and cdk6 activity can substitute for *RB* mutations, and define the "pRb pathway" of genetic events that have the identical phenotypic consequence of pRb inactivation and inappropriate proliferation. A further oncogenic consequence of excess cyclin D/cdk4(6) expression is sequestration of p21 and p27 with consequent elevated activity of cyclin E/cdk2, a function that may be an extension of a physiological role of cyclin D/cdk4(6) complexes [14, 26-28].

Inconsistent with the apparent functional redundancy of each of these genetic events in the pRb pathway in cancer is the fact that many cells express two or three D-type cyclins and both cdk4 and cdk6. Furthermore, varying tumor types have been found to preferentially alter certain components of this pathway and to specifically increase activity of either cdk4 or cdk6, but not both. For instance, cdk4 seems to be specifically targeted in melanoma [24, 29] while increased cdk6 activity was found in squamous cell carcinomas [30] and neuroblastomas [31] without alteration of cdk4 activity. These findings suggest that cdk4 and cdk6 may not be entirely redundant in all cells and may be individually targeted by distinct cell-type specific mitogenic signals directing their temporal or substrate-specific activities.

To better understand the role of cdk6 as an oncogene, we began by studying the requirements for proliferation caused by ectopic cdk6 expression.

Cdk6-transfected cells demonstrated accelerated transit through the G1 phase of the cell cycle. This effect of cdk6 on cell cycle progression was dependent on the INK4 binding domain, since a cdk6 mutant (R31C) unable to associate with INK4 proteins did not show G1 acceleration. In addition, this mutant protein failed to accumulate in the nucleus, suggesting that nuclear localization and function of cdk6 is dependent on the INK4 interaction domain. Further, catalytic activity of cdk6 was required for G1 acceleration in this assay, since a catalytically inactive NFG mutant slowed S phase entry rather than accelerated it, despite an ability to form complexes with cyclin D1 and p27. Thus, cdk6 activity is limiting for G1-to-S phase progression even in tumor cells like U2OS, which lack p16INK4a, strongly supporting a role for cdk6-specific phosphorylation events in G1 progression.

Experimental Procedures

Expression vectors, transfection procedures and cell lines. The kinase expression plasmids pCMVcdk6 and HA-tagged cdk6, pCMVcdk6HA, pCMVcdk6NFG, the vector pCMVneobam and the CD20-encoding plasmid, pCMVCD20, were kindly provided by Dr. Sander van den Heuvel [32]. The vector containing these cDNA's pCMVBamNeo has been previously described [33]. U2OS cells were transiently transfected with 15 micrograms (μg) kinase-expressing plasmid plus 5 μgs pCMVCD20 (where appropriate) and sheared herring sperm DNA to a total of 30 μgs by calcium phosphate precipitation essentially as described by Chen and Okayama [34]. DNA precipitates remained on the cell monolayer for 17 hours and cells were harvested 24 hours after removal of DNA precipitates unless otherwise noted. U2OS cells were maintained in 10% fetal calf serum (FCS) at 5% CO_2.

Analysis of cell cycle distribution. For fluorescence-activated cell sorting (FACS) experiments, transfected U2OS cells were harvested in PBS with .1% EDTA at 24 hours after removal of DNA precipitates. Cells were then stained with fluoresceine isothiocyanate (FITC) conjugated antibody to human CD20 (Pharmingen), ethanol fixed and stained with propidium iodide for DNA content. Cell cycle distribution was analyzed by flow cytometry of CD20-positive (FITC positive) cells using a Coulter Cytometer and Multicycle DNA-analysis. In FACS studies of nocodazole treated cells, nocodazole was added at 24 hours after removal of DNA precipitates to a final concentration of 100 ng/ml for 18 hours. In FACS studies following mitotic shake, mitotic fractions were harvested by gentle pipeting followed by centrifugation at 1000 rpm at 25°C for 5 mins. Cells were washed three times with media to remove nocodazole and replated in 10% FCS media. Cells were harvested at time points indicated and prepared for FACS as described above.

BrdU and immunofluorescence. In 5-bromo-2'-deoxy-uridine (BrdU) incorporation experiments nocodazole was added to 100 ng/ml approximately 5 hours after removal of DNA precipitates and remained on cells 18 hours. BrdU was added to a final concentration of 10 micromolar (μM) at time points indicated. Coverslips from BrdU experiments were fixed in 70% ethanol, 50 millimolar (mM) glycine, pH 2.0 and incubated with BrdU monoclonal antibody (Bohringer-Mannheim) and polyclonal peptide antibodies to cdk6 (Santa-Cruz, C-21) for 60 mins at 37°C. Secondary antibodies rhodamine conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and fluoresceine conjugated anti-mouse (Bohringer-Mannheim), were incubated 30 mins at 37°C. Coverslips were mounted in Fluoromount. For timecourses, mitotic shake was performed as described above and coverslips fixed at indicated time points. For

immunofluorescence without BrdU, coverslips were stained in methanol followed by acetone. Immunofluorescence was performed with antibodies indicated above as well as cdk6 polyclonal sera of Meyerson [4] and cdk6 monoclonal sera Ab-3 (Neomarkers, Fremont, CA) for 60 mins at 37°C. Secondary antibody staining was performed as described above or with fluoresceine-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). In relevant cases, cells were counterstained by Hoechst stain. All photography was performed on a Leica microscope with Sony digital imaging.

Biochemical assays. For immunoblot and immunoprecipitation experiments, 1.25 X 10^6 U2OS cells were transfected as described above, harvested 24 hours after removal of DNA precipitates, washed twice with phosphate buffered saline and harvested in E1A lysis buffer (ELB) (250mM NaCl, 50mM Hepes pH 7.0, 5mM EDTA, 0.1%NP40). Extracts were incubated 20 mins on ice with mixing and clarified by centrifugation for 20 mins at 4°C. Proteins were separated on polyacrylamide denaturing gels, transferred to supported nitrocellulose (Gibco BRL) and blotted using antisera as noted.

Immunoprecipitations were performed with 2μg polyclonal cdk6 antisera C-21. Transfected U2OS cells were lysed in ELB buffer as described above and 200 μg (cyclin D1) or 400 μg (p18, p27) of extract was immunoprecipitated for 60 mins at 4°C with mixing. 35μl of protein A-Sepharose swollen beads were added for an additional 30 min, washed 4 times with 1 ml ELB and separated on denaturing acrylamide gels. Antibodies used include: p18 polyclonal N-20 1:3000 (Santa Cruz), p27 monoclonal antibody 1:2500 (Transduction Labs), cyclin D1 monoclonal DCS-6 1:200 (Neomarkers).

For kinase assays, SAOS-2 cells at 80% confluency were transfected with 10μgs pCMVD1 and 10μgs pCMVcdk6HA or cdk6mutantHA in the pCMV

vector by calcium phosphate as described above. DNA precipitates remained on cells 10 hours and were harvested 36 hours after removal of DNA precipitates. Cells were harvested in D-IP Kinase Buffer (50mM Hepes pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, .1% Tween 20, 10% glycerol with protease inhibitors aprotinin, leupeptin and pefablock and phosphatase inhibitors sodium orthovanadate (100μM), sodium flouride (10mM), and betaglycerophosphate (10mM)) and incubated on ice for 20 mins with gentle mixing. Lysates were clarified by centrifugation at 4°C for 10 mins. 100μl of 12CA5 antibody was preincubated with 30μl swollen protein A sepharose beads at least 1 hour at 4°C with mixing. 100μg of cell lysate was added for an additional hour at 4°C with mixing. Beads were washed 3 times with D-IP buffer and 3 times with Kinase Reaction Buffer (250mM Hepes, pH 7.2, 50mM $MgCl_2$, 25mM $MnCl_2$, 1mM DTT). Kinase reactions were performed with HA-immunoprecipitated extracts at 37°C for 30 mins in Kinase Reaction Buffer with 100μM ATP, 10uCi gamma ^{32}P ATP and 0.5 μg C-terminal GST-Rb (aa769 to 921) (Santa-Cruz Biotechnology) as substrate. Reactions were stopped by addition of protein sample buffer with 10% betamercaptoethanol and placed on ice. Samples were boiled and separated on 12.5% denaturing acrylamide gel, Coomassie Brilliant Blue stained to ensure equal loading and addition of Rb substrate, and exposed to film overnight.

Results

Cdk6 causes increased S-phase of transfected cell populations.

The effects of ectopic expression of cdk6 on cell cycle progression was determined by fluorescence-activated cell sorting (FACS) studies in transfected U2OS cells. These human osteosarcoma cells produce wild-type pRb but lack $p16^{INK4a}$, a defect that is thought to allow constitutive phosphorylation and

inactivation of pRb. In light of this, it was surprising to observe that cells transfected with plasmid encoding cdk6 consistently showed a higher percentage of S-phase cells than did vector transfected cells in the same experiment (Figure 1A). To confirm that this increase in DNA content measured by FACS truly reflected an increase in S-phase cells, transfected cell cultures were also analyzed using bromodeoxyuridine (BrdU) incorporation as a measure of S-phase. U2OS cells transfected with pCMVcdk6 or pCMVvector were pulsed with BrdU, fixed and subjected to indirect immunofluorescence using both anti-BrdU and anti-cdk6 antibodies. Transfected cells were identified as those that demonstrated intense fluorescence with anti-cdk6 antibody and were scored as either BrdU-positive or BrdU-negative. The results of at least two independent transfections ($\geq$700 kinase positive cells counted) demonstrated that 34% of vector-transfected cells were BrdU positive while in the same experiment 50% of cdk6-transfected cells were BrdU positive (Figure 1B). These data closely match the results of FACS analysis shown in figure 1A and indicate that cells transfected with cdk6 showed a statistically significant ($p<.05$) increase in the percentage of S-phase population as compared to vector-transfected cells.

cdk6 shortens the G1 interval of transfected U2OS cells.

The observed increase in S-phase cells conferred by cdk6 could result from either an S-phase cell cycle block or from decreased transit time through G1 or G2/M. To distinguish between these possibilities, transfected cells were treated with the mitotic inhibitor nocodazole. In the presence of nocodazole, an S-phase delay would reduce the number of cells able to enter G2/M. However, if the increased S-phase population was due to a shortening of G1 phase (or G2/M phase) cells would arrest in mitosis under nocodazole treatment. At 24 hours after removal of DNA precipitates, parallel sets of transfected U2OS cells

were either harvested or treated with nocodazole for 18 hours. A p16 control for nocodazole arrest demonstrated that p16 transfected cells maintained a G1 phase peak as expected with a G1-arresting inhibitor (Figure 2A). In the same experiment cdk6-transfected cells accumulated in mitosis in the presence of nocodazole, indicating that the S-phase increase seen by FACS and BrdU incorporation studies (Figure 1) was not due to a profound S-phase delay but was more likely a result of a decreased G1 or G2/M transit time.

To determine if ectopic cdk6 expression decreased G1 phase transit time, FACS analysis was performed on synchronized cell populations. Transfected U2OS cells were treated with nocodazole for 18 hours followed by shaking and replating in nocodazole-free medium. The cells were harvested at 4 and 8 hours after mitotic shake and DNA profiles of CD20 positive cells were obtained by FACS as shown in Figure 2B. At 4 hours after mitotic shake, cdk6-transfected cells showed a synchronized 2N DNA profile indistinguishable from that of vector transfected cells, indicating a similar entry into G1 phase. Interestingly, at 8 hours post mitotic shake, cdk6 transfected cells showed a shift toward S-phase (increased DNA content) as compared with vector transfected cells. Together these experiments indicate that cdk6-transfected U2OS cells pass through G1 phase faster than vector-transfected cells, demonstrating a cdk6-dependent acceleration of G1 transit in U2OS cells. Also shown in figure 2B are DNA profiles of a mutant form of cdk6, cdk6R31C. cdk6R31C contains a mutation of the arginine residue corresponding to arg 24 in cdk4. Mutation of this residue (arg 24 to cys) in cdk4 was identified in a human melanoma and prevents binding to the kinase inhibitor, p16 [24]. Interestingly, cdk6R31C did not demonstrate the shift toward S phase seen with the wildtype cdk6, suggesting a role for the INK4 binding domain in the G1 acceleration function of cdk6. The lack of G1 acceleration exhibited by cdk6R31C could be due to an

unexpected loss of catalytic activity or to the disruption of another property of cdk6 required for this G1 acceleration function in U2OS cells.

Biochemical characterization of cdk6 mutants.

The acceleration of G1 phase caused by ectopic expression of cdk6 could be the result of direct catalytic activity of the introduced kinase subunit phosphorylating substrates like pRb to shorten G1 phase. Alternatively, excess kinase subunits could titrate inhibitory proteins to allow activation of other cdks and concomitant cell cycle advance. Titration of inhibitory proteins has been observed to occur upon introduction of both functional and nonfunctional kinases in another system, apparently through titration of p21 [28], and cyclin D/cdk4(6) complexes have been suggested to sequester p27 in the absence of anti-mitogenic signals [26, 27]. In an effort to determine the properties of cdk6 required to accelerate G1 progression in U2OS cells, a series of cdk6 mutants compromised in their ability to bind to INK4 protein (cdk6R31C), hydrolyze ATP (cdk6NFG) [32], or both (cdk6R31CNFG) were used in cell cycle analyses. The biochemical characterization of these mutant proteins is presented in Figure 3. Importantly, all cdk6 mutants consistently showed approximately equal protein levels in transfected U2OS lysates as shown by immunoblot in Figure 3A. Consistent with the predicted result, we found that the R31C mutation prevented interaction with $p16^{INK4a}$ (data not shown) and $p18^{INK4c}$ in transfected U2OS lysates (Figure 3B), as has also been observed in breast cancer cell lines [35]. The $p18^{INK4c}$ interaction is particularly relevant to U2OS cells since these cells lack $p16^{INK4a}$, yet express detectable levels of $p18^{INK4c}$ bound to cdk6 (data not shown). Disruption of INK4 binding occurred whether the mutation was present alone (cdk6R31C), or in combination (cdk6R31CNFG) with the catalytically inactive mutation (Figure 3B). Importantly, the R31C

mutation does not disrupt the ability of cdk6 to bind cyclin D1 or p27 in immunoprecipitations of transfected U2OS extracts (Figure 3C, 3D). In these experiments, immunoblots were stripped and reprobed with anti-cdk6 antibody to ensure equivalent levels of cdk6 protein were compared in binding studies. Thus, the cdk6R31C mutation that corresponds to the tumor-derived cdk4R24C mutation [24] specifically disrupts cdk6 binding to INK4 proteins without altering interaction with other known cdk6 partners.

To ensure that the R31C mutant form of cdk6 retained catalytic activity, the cdk6 mutants were also examined for kinase activity in transfected SAOS-2 cells (used in these assays because they contain low-levels of endogenous cyclin D1 and cdk6). As shown in Figure 3E, when co-transfected with cyclin D1 and immunoprecipitated with antibody to the HA tag, HAcdk6 phosphorylated the C-terminal GST-Rb substrate. Conversely, cdk6NFG containing the kinase inactivating mutation showed no kinase activity above vector-transfected background. Significantly, cdk6R31C-transfected extracts reproducibly showed *in vitro* kinase activity greater than that observed with wildtype cdk6 extracts, as expected for a mutant that can evade the $p16^{INK4a}$ present at high level in SAOS-2 cells. Anti-HA immunoblots of these extracts confirmed that the level of cdk6R31C was at or below the level of wildtype cdk6 protein (not shown). Interestingly, the double mutant cdk6R31CNFG had a slightly elevated activity relative to the inactive NFG mutant. This result suggests that a low level cdk6NFG activity is unmasked in cdk6R31CNFG by the disruption of INK binding.

Thus, the cdk6R31C mutation disrupts INK4 protein binding but does not disrupt intrinsic catalytic activity of this cdk6 protein, similar to studies demonstrating retention of kinase activity by the $p16^{INK4a}$-binding defective mutant of cdk4, cdk4R24C [24]. Importantly, these binding studies and kinase

assays also indicate that these mutations are not causing gross structural alterations in the cdk6 protein. These reagents are thus ideal for assessing the potential roles of catalytic activity and INK4 titration in the cdk6-mediated acceleration of G1 phase in transfected U2OS cells.

cdk6 mutants do not accelerate G1 phase.

The cdk6 mutants described above were used to further examine cdk6 function in G1 acceleration of U2OS cells. To test the ability of mutant forms of cdk6 to decrease G1 transit time, BrdU incorporation was used to measure S-phase entry by the cdk6 mutants. In these experiments BrdU was added to transfected U2OS cells 4 hours after mitotic shake and BrdU incorporation was measured at 5, 7, and 10 hours after mitotic shake. The results of these experiments in which cumulative BrdU incorporation was measured are presented in Figure 4. Consistent with the results shown in Figure 2B, cdk6 wildtype transfectants accumulated BrdU positive (S phase) cells faster than did vector transfected controls, such that at 10 hours 60% of wildtype cdk6 transfectants were BrdU positive but only 39% of vector control cells were BrdU positive. Interestingly, the kinase inactive mutant, cdk6NFG, showed a slower entry into S-phase as compared to the vector transfected control. In fact, cdk6NFG showed a greatly decreased percent of cells in S-phase at all timepoints measured (27% at 10 hrs). FACS analysis after nocodazole treatment suggested that this decrease in S-phase entry was due to a G1 delay since a significant G1 fraction persisted in nocodozole treated cells (not shown). A similar delay to S-phase entry has been observed with cdk4NFG [36]. Thus, catalytic activity and not soley inhibitor titration appears to be required for the G1 acceleration observed with wildtype cdk6 since cdk6NFG was incapable of increasing the S phase fraction of transfected cells, but is fully capable of inhibitor interaction.

Because the result above suggests that kinase activity intrinsic to cdk6 is key to accelerating G1 phase in transfected U2OS cells, the cdk6R31C mutant was expected to give an increase in S phase cells equal to or greater than that conferred by wild-type cdk6, given the ability of cdk6R31C to phosphorylate pRb and avoid interaction with INK4 proteins. However, consistent with results in Figure 2, cdk6R31C did not show the G1 acceleration typical of wildtype cdk6, as might be expected if $p18^{INK4c}$ acts to limit cdk6 activity in these cells. In fact, both cdk6R31C (41%) and cdk6R31CNFG (38%) show significantly fewer cells in S-phase than did cdk6wt (60%) at 10 hours after release from mitotic block. The values for the cdk6R31C and cdk6R31CNFG mutants were similar to that of the vector transfected control (39%). Thus, intrinsic kinase activity appears to be necessary but not sufficient for the increase in S-phase population caused by cdk6wt since cdk6R31C, which has demonstrated in vitro kinase ability, cannot accelerate G1 phase. Further, the R31C mutation in cdk6R31CNFG nullifies the S-phase inhibitory effect seen after introduction of cdk6NFG, suggesting a critical role for the R31 residue in cdk6 function.

Cell cycle effects correlate with nuclear localization.

Previous studies have shown that the subcellular localization of cdk4 may influence its interaction with the CIP and INK family of inhibitors [27]. In addition, both cdk4 and cdk6 have been observed to localize to the cytoplasm in a variety of cell types [37-39]. Thus, we wished to test the hypothesis that the inability of the cdk6 mutants to accelerate G1 phase may be in part due to differential localization within the cell. Transfected U2OS cells were synchronized using nocodazole and analyzed by indirect immunofluorescence for cdk6. Repeatedly, the mutant forms of cdk6 that failed to bind INK4 proteins (cdk6R31C and cdk6R31CNFG) demonstrated greatly decreased nuclear staining

as compared to cdk6wt and cdk6NFG at 8-10 hours after mitotic shake (Figure 5). These results were repeated in at least 3 separate transfections and with two distinct staining methods using both polyclonal and monoclonal antibodies (Figure 5).

The decrease in nuclear staining observed in cdk6R31C and cdk6R31CNFG transfectants was also observed in asynchronous populations at 24 hours after removal of DNA precipitates but in these populations the percent of cdk6R31C and cdk6R31CNFG mutants with predominantly cytoplasmic staining was lower than the percent seen in a synchronous population (shown in figure 5), suggesting that the localization of these kinases is cell cycle regulated.

Together, these studies demonstrate that the R31C mutation affects compartmentalization of cdk6 as well as the ability to interact with INK4 proteins. R31C mutants showed a remarkable decrease in nuclear staining particularly at timepoints predicted to be at or near the G1/S boundary. This difference in compartmentalization directly correlated with the inability of the same mutants to accelerate G1 phase of the cell cycle and suggested a role for INK4 protein binding domain in the generation of functional, nuclear cdk6 complexes.

Discussion

The D-cyclin-dependent kinases cdk4 and cdk6 share pRb as their only proven physiological substrate and both can act as oncogenes in human tumors that retain pRb, suggesting redundancy of function. Nevertheless, the fact that many cells express both cdk4 and cdk6, coupled with the observation that some tumor types specifically activate only one of these kinases, suggests that each kinase may play a unique role in cell cycle progression. Previous experiments have identified numerous, tumor-derived cdk4 mutants that fail to interact with

$p16^{INK4a}$, suggesting that this kinase acts as an oncogene by evading inhibition by INK4 proteins. This may in turn result in direct modification of substrates by high levels of cdk4 activity or may produce and indirect effect through increased p21/p27 titration. The results presented here demonstrate that cdk6 can accelerate G1 phase transit when ectopically expressed in U2OS cells even though these cells do not express $p16^{INK4a}$.

Expression of cdk6 resulted in increased S-phase as measured by FACS and BrdU incorporation. Studies of cells released from mitotic block indicated that the observed increase in S-phase cells resulted at least in part from decreased transit time through G1 phase of the cell cycle. Although we cannot rule out an accompanying lengthening of S phase, nocodazole treated, cdk6-transfected cells did accumulate in M phase, suggesting that any effect on S phase transit time was not large. Surprisingly, the accelerated G1 phase did not require co-transfection of the kinase activating partner, cyclin D. G1 acceleration by cdk6 in the absence of increased cyclin D is consistent with a model in which the supply of cyclin D is not the only rate-limiting step in kinase activation. Indeed, cyclin D1 levels are quite stable across the cell cycle in many proliferating cells, unlike cyclins A, E, and B. Thus, while cyclin D complex formation is obviously a critical step in kinase activation, it may not be the rate limiting step in cultures of proliferating cells.

The availability of the kinase may be particularly limiting for cyclin D1/cdk4 (cdk6) complexes, since the INK4 family of inhibitors act as competitors with cyclin D for cdk4 and cdk6 binding. Thus, $p16^{INK4a}$-nonbinding mutants of cdk4 are oncogenic by virtue of their insensitivity to growth arrest (senescence) signals presumably transduced through $p16^{INK4a}$ (Hara et al., 1996; Serrano et al., 1997; McConnell et al., 1998; Wölfel et al., 1995). In light of this, the ability of cdk6 to accelerate G1 in U2OS cells is

somewhat surprising, given that U2OS cells lack $p16^{INK4a}$, and thus are thought to be able to phosphorylate and inactivate pRb without inhibition of cdk4 or cdk6 activity. Nevertheless, our results suggest that cdk6 is limiting for cell cycle progression despite the absence of $p16^{INK4a}$. This is consistent with the observed ability of excess cdk4 to increase the proliferation of astrocyte cell lines lacking $p16^{INK4a}$ [40]. One possibility arising from such observations is that cdk6 can act "noncatalytically" in this system by sequestering p21 or p27 away from cdk2. Such a role for cyclin D/cdk4(6) complexes is strongly supported by experiments demonstrating that $p15^{INK4b}$ and $p16^{INK4a}$ can cause redistribution of p21 and p27 from cyclin cdk4(6) complexes to those containing cdk2, thereby augmenting cell cycle arrest [26, 27, 41, 42].

The role of cyclin D/cdk4(6) complexes as "sinks" for p21 and p27 suggests that catalytic activity of cdk6 would be dispensible for G1 acceleration if titration alone were sufficient to shorten G1. Our results using cdk6NFG, which is catalytically inactive yet still able to bind D cyclins and p27, argue that such p21 and p27 titration is not responsible for cdk6-mediated G1 acceleration in U2OS cells. cdk6NFG was completely incapable of shortening G1 phase in these experiments, and indeed it detectably delayed S phase entry as determined by BrdU incorporation following release from nocodazole (Figure 4). These results strongly argue that catalytic activity of cdk6 is required for the observed effects on cell cycle progression in U2OS cells and inhibitor sequestration is not sufficient for this effect.

It is possible that kinase inhibition by other members of the INK4 family limit proliferation in cultured cells lacking $p16^{INK4a}$. For example, G1 length may be partly determined by the ratio of endogenous cdk6 to $p18^{INK4c}$ [43], which is expressed in U2OS cells, and excess cdk6 would thus result in a shortened G1 phase. This model predicts that elimination of INK4c binding by

the R31C mutation, which is analagous to the oncogenic R24C mutation in cdk4, would enhance the G1 acceleration function of cdk6. However, our results using cdk6R31C stand in direct contradiction to this, since R31C is completely unable to alter G1 phase in transfected U2OS cells. Because this mutation, like the NFG mutation, does not disturb properties of cdk6 such as cyclin D1 or p27 binding, this result further argues against a role for inhibitor titration in the G1 accleration function of cdk6. Indeed, because cdk6R31C can be activated by cyclin D1 in cotransfected cells, these results suggest that the N-terminus of cdk6 may be involved in the proper function of cdk6 within cells (but not *in vitro*), perhaps at the level of substrate recognition or compartmentalization.

Published reports indicate that cdk4 and cdk6 is indeed regulated at the level of subcellular localization [13, 27, 38]. Consistent with these studies, data shown here demonstrate that cdk6 localizes to both the nucleus and the cytoplasm while cdk6R31C preferentially localizes to the cytoplasm. Synchronized U2OS cells show a striking lack of cdk6R31C and cdk6R31CNFG protein in the nucleus in late G1 phase. Recently it has been shown that cytoplasmic cdk4 and cdk6 exist primarily in inactive complexes with cdc37 and hsp90 or (in T cells) with $p19^{INK4d}$ [37, 39]. In light of this, the localization pattern of the cdk6R31C protein presents an apparent paradox. Why is an INK4-binding defective cdk6 protein preferentially localized in the cytoplasm if the major function of the cytoplasmically-localized INK4 protein (in this case, specifically $p18^{INK4c)}$ is to anchor kinases in an inactive state? We suggest that the N terminus of cdk6 is critically involved in the dissolution of cytoplasmic complexes, and may be a binding site for proteins that serve to promote translocation of cdk6 to the nucleus. It is possible that such proteins resemble INK4 proteins, or it may even be the case that INK4 proteins themselves could promote nuclear entry of cdk6 under certain circumstances, since INK4 proteins

can compete with cdc37/hsp90 for binding to cdk4 and cdk6 ([44] and M Grossel, P. Hinds unpublished observations). The precise role of the cdk6 N-terminus in subcellular localization is currently under investigation, but whatever the mechanism, maintenance of the cdk6R31C mutant in the cdc37 complex predicts a persistence of cytoplasmic localization and a functionally inactive kinase. In fact, this is precisely the phenotype observed with cdk6R31C: An increased cytoplasmic retention (Figure 5) and a loss of function in either G1 acceleration (cdk6wt) or G1 retardation (cdk6NFG) (Figure 4), despite greater than wildtype catalytic activity of cdk6R31C in *in vitro* kinase assays (Figure 3).

The requirement for cdk6 nuclear localization in G1 acceleration, its likely regulation in the cell cycle, and the novel role of the N-terminus in this localization raise the possibility of the existence of a cdk6 regulatory pathway that may result in differential activity of cdk4 and cdk6 in the same cell. Indeed, the fact that cdk4R24C has been reported to be hyperactive but cdk6R31C is inactive in cell cycle progression suggests that these kinases may be subject to discrete activation programs. In the case of cdk6, production of active complexes may require the presence of a factor that interacts with the N-terminus and which is itself subject to cell cycle regulation. If this factor is not required by cdk4, the two kinases could respond differently to extracellular signals, and this in turn could favor activation of one kinase versus another in tumor cells. Clearly a better understanding of the role of the cdk6 N-terminus in functional regulation and the identification of putative activating factors that interact with this domain are required to fully understand the role of cdk6 in cancer cells.

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References

1. Matsushime, H., *et al.* (1992) *Cell* **71,** 323-334
2. Meyerson, M., *et al.* (1992) *EMBO* **11,** 2909-2917
3. Matsushime, H., *et al.* (1994) *Mol. Cell. Biol.* **14,** 2066-2076
4. Meyerson, M. and Harlow, E. (1994) *Mol. Cell. Biol.* **14,** 2077-2086
5. Weinberg, R. (1995) *Cell* **81,** 323-330
6. Morgan, D. (1995) *Nature* **474,** 131-134
7. El-Deiry, W.S., *et al.* (1993) *Cell* **75,** 817-825
8. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993) *Cell* **75,** 805-816
9. Xiong, Y., *et al.* (1993) *Nature* **366,** 701-704
10. Polyak, K., *et al.* (1994) *Genes & Dev.* **8,** 9-22
11. Toyoshima, H. and Hunter, T. (1994) *Cell* **78,** 67-74
12. Zhang, H., Hannon, G., and Beach, D. (1994) *Genes & Dev* **8,** 1750-1758
13. LaBaer, J., *et al.* (1997) *Genes & Dev.* **11,** 847-862
14. Cheng, M.G., *et al.* (1999) *EMBO J.* **18,** 1571-1583
15. Brotherton, D., *et al.* (1998) *Nature* **395,** 244-250.
16. Russo, A., Tong, L., Lee, J., Jeffrey, P., and Pavletich, N. (1998) *Nature* **395,** 237-243
17. Peter, M. and Herskowitz, I. (1994) *Cell* **79,** 181-184
18. Motokura, T., *et al.* (1991) *Nature* **350,** 512-515
19. Khatib, Z., *et al.* (1993) *Cancer Res.* **53,** 5535-5541
20. Nobori, T., *et al.* (1994) *Nature* **368,** 753-756
21. Tsujimoto, Y., *et al.* (1994) *Science* **224,** 1403-1406
22. Kamb, A., *et al.* (1994) *Science* **264,** 436-440

23. Ladanyi, M., *et al.* (1995) *J. of Pathol.* **175,** 211-217

24. Wolfel, J., *et al.* (1995) *Science* **269,** 1281-1284

25. Costello, J.F., *et al.* (1997) *Cancer Research* **57,** 1250-1254

26. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) *Genes & Dev.* **9,** 1831-1845

27. Reynisdottir, I. and Massague, J. (1997) *Genes & Dev.* **11,** 492-503

28. Latham, K.M., Eastman, S.W., Wong, A., and Hinds, P.W. (1996) *Mol. Cell. Biol.* **16,** 4445-4455

29. Zuo, L., *et al.* (1996) *Nature genet.* **12,** 97-99

30. Timmermann, S., Hinds, P.W., and Munger, K. (1997) *Cell Growth and Differentiation* **8,** 361-370

31. Easton, J., Wei, T., Lahti, J., and Kidd, V. (1998) *Cancer Research* **58,** 2624-2632

32. van den Heuvel, S. and Harlow, E. (1993) *Science* **262,** 2050-2054

33. Baker, S.J., Markowitz, S., Fearon, E.R., Wilson, J.K.V., and Vogelstein, B. (1990) *Science* **249,** 912-915

34. Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745-2752

35. Lapointe, J., Lachance, Y., Labrie, Y., and Labrie, C. (1996) *Cancer Research* **56,** 4586-4589

36. Jiang, H., Chou, H., and Zhu, L. (1998) *Mol. Cell. Biol.* **18,** 5284-5290

37. Stepanova, L., Leng, X., Parker, S.B., and Harper, J.W. (1996) *Genes & Dev.* **10,** 1491-1502

38. Nagasawa, M., Melamed, I., Kupfer, A., Gelfand, E., and Lucas, J. (1997) *J. of Immunology* **158,** 5146-5154

39. Mahony, D., Parry, K., and Lees, E. (1998) *Oncogene* **16,** 603-611

40. Holland, E.C., Hively, W.P., Gallo, V., and Varmus, H.E. (1998) *Genes & Dev.* **12,** 3644-3649

41. Parry, D., Mahony, D., Wills, K., and Lees, E. (1999) *Mol. Cell. Biol.* **19,** 1775-1783

42. McConnell, B.B., Gregory, F.J., Stott, F.J., Hara, E., and Peters, G. (1999) *Mol. Cell. Biol.* **19,** 1981-1989

43. Zindy, F., Quelle, D., Roussel, M., and Sherr, C. (1997) *Oncogene* **15,** 203-211

44. Lamphere, L., *et al.* (1997) *Oncogene* **14,** 1999-2004

Figure Legends

Figure 1: DNA profiles and BrdU incorporation of vector and cdk6 transfected cells. A: U2OS cells were transfected with indicated plasmids and harvested for FACS analysis. Cells were incubated with FITC-conjugated CD20 antibody to distinguish transfected populations, fixed and stained with propidium iodide for DNA content. At least 1,800 CD20-positive events were counted per histogram. Results are representative of those found in at least three independent experiments. B: BrdU incorporation for vector and cdk6 transfected cells. Values represent the average of at least two independent experiments for which at total of at least 700 events were counted.

Figure 2: Cell cycle profiles of transfected cells. FACS analysis of transfected U2OS cells. Cells were incubated with FITC-conjugated CD20 antibody to distinguish transfected populations, fixed and stained with propidium iodine for DNA content. A: FACS profiles of p16 and cdk6 transfected U2OS cells either untreated or nocodazole treated, as indicated. B: DNA profiles of nocodazole synchronized cells transfected with vector (solid line), cdk6 (dotted

line), or cdk6R31C (bold line) and harvested at 4 and 8 hours following mitotic shake and replating.

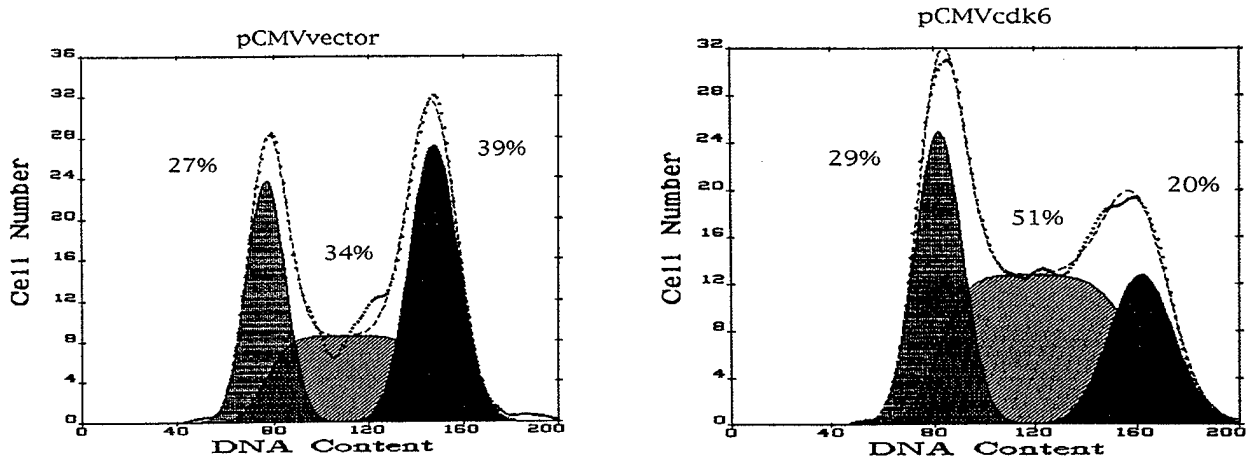
Figure 3: Biochemical characterization of cdk6 and cdk6 mutants. A: Direct immunoblot of 20 µg of transfected U2OS cell lysate using polyclonal anti-cdk6 antibody C-21. B: cdk6 immunoprecipitation of 400µg transfected cell lysate immunoblotted with polyclonal p18 antibody, then reprobed with cdk6 antibody, as noted. C: cdk6 immunoprecipitation of 200µg U2OS lysate immunoblotted with monoclonal cyclin D1 antibody and cdk6 antibody, as noted. D: cdk6 immunoprecipitation of 400µg cell lysate immunoblotted with polyclonal p27 antibody and reprobed with cdk6 antibody, as noted. E: In vitro kinase assay. SAOS-2 cells were transfected with cyclin D1 (lanes 1 and 3-7) or its empty vector (lane 2), and the indicated kinase or its vector. Lysates from each transfection were tested for their kinase activity using C-terminal GST-Rb substrate (aa769-921).

Figure 4: BrdU incorporation of U2OS cells expressing cdk6 mutants. Graph of BrdU incorporation of kinase positive cells as determined by immunofluorescence. BrdU incorporation of transfected U2OS cells harvested at 5, 7, or 10 hours after mitotic shake. At least 120 vector transfected (closed squares), cdk6wt (closed circles), cdk6R31C (closed triangles), cdk6R31CNFG (open triangles), and cdk6NFG (open squares), positive cells were scored as BrdU positive or BrdU negative at each time point.

Figure 5: Localization of kinases. Indirect immunofluorescence of transfected cdk6 or mutant forms of cdk6. A: Cells were fixed at 10 hours after synchronization and stained with polyclonal antibody to cdk6 (C-21). B:

Cells were fixed at 9 hours after synchronization and stained with monoclonal antibody to cdk6 (Ab-3).

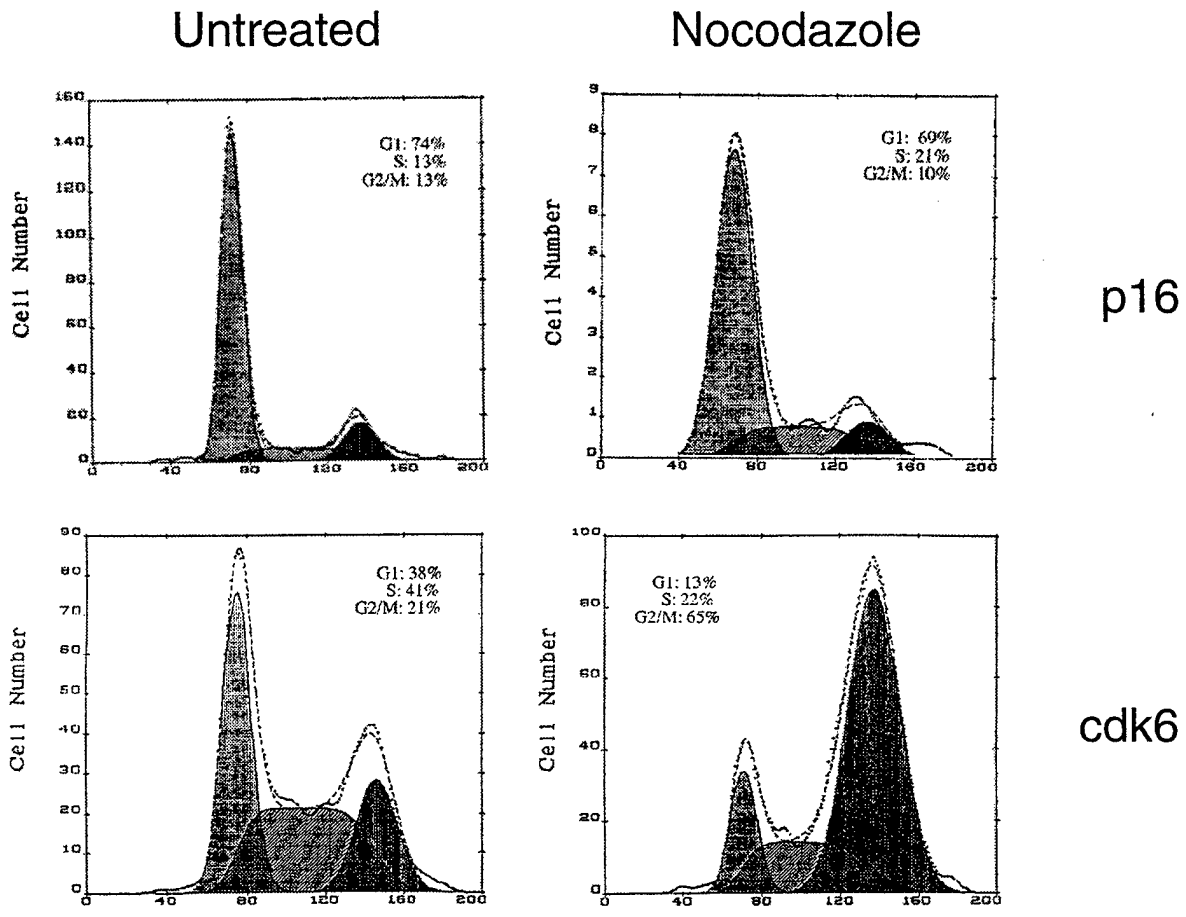
A



B

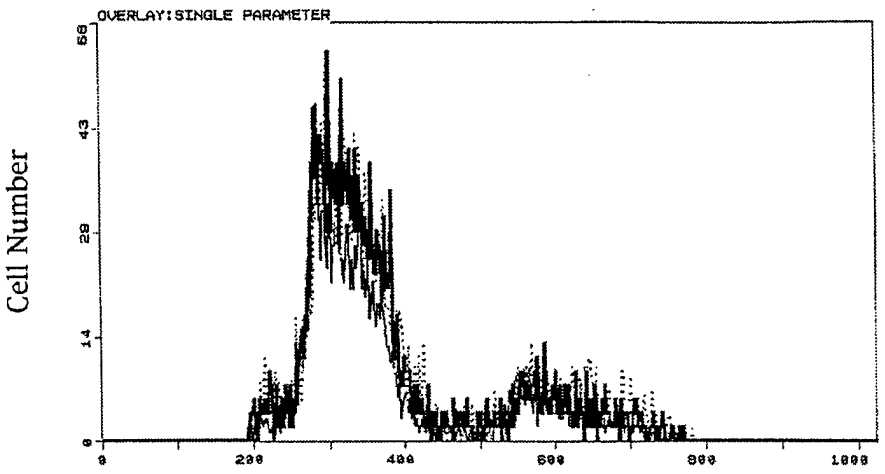
plasmid transfected	cells counted	Kinase + Brdu +	Kinase + Brdu -
pCMVvector	732	34%	66%
pcmvCDK6	902	50%	50%

A

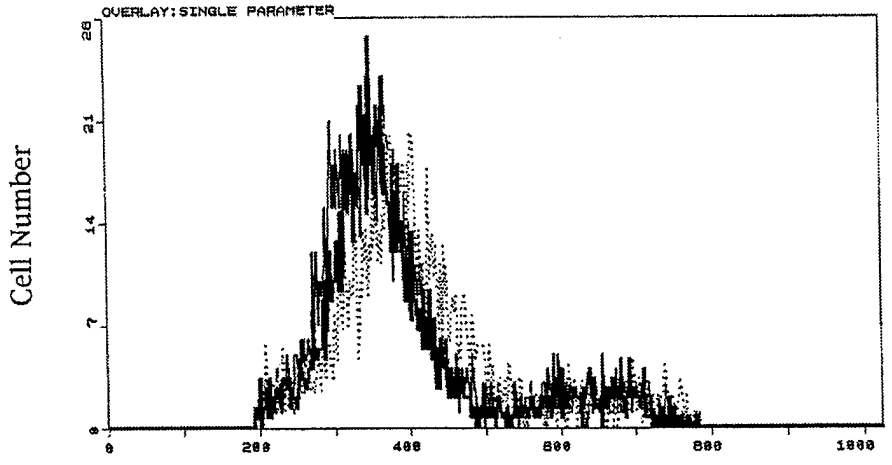

Untreated
Nocodazole
Cell Number
G1: 74%
S: 13%
G2/M: 13%
G1: 69%
S: 21%
G2/M: 10%
p16
G1: 38%
S: 41%
G2/M: 21%
G1: 13%
S: 22%
G2/M: 65%
cdk6

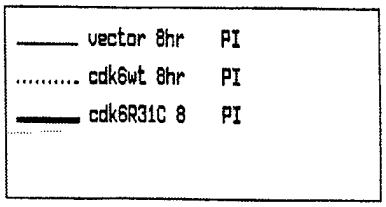
B

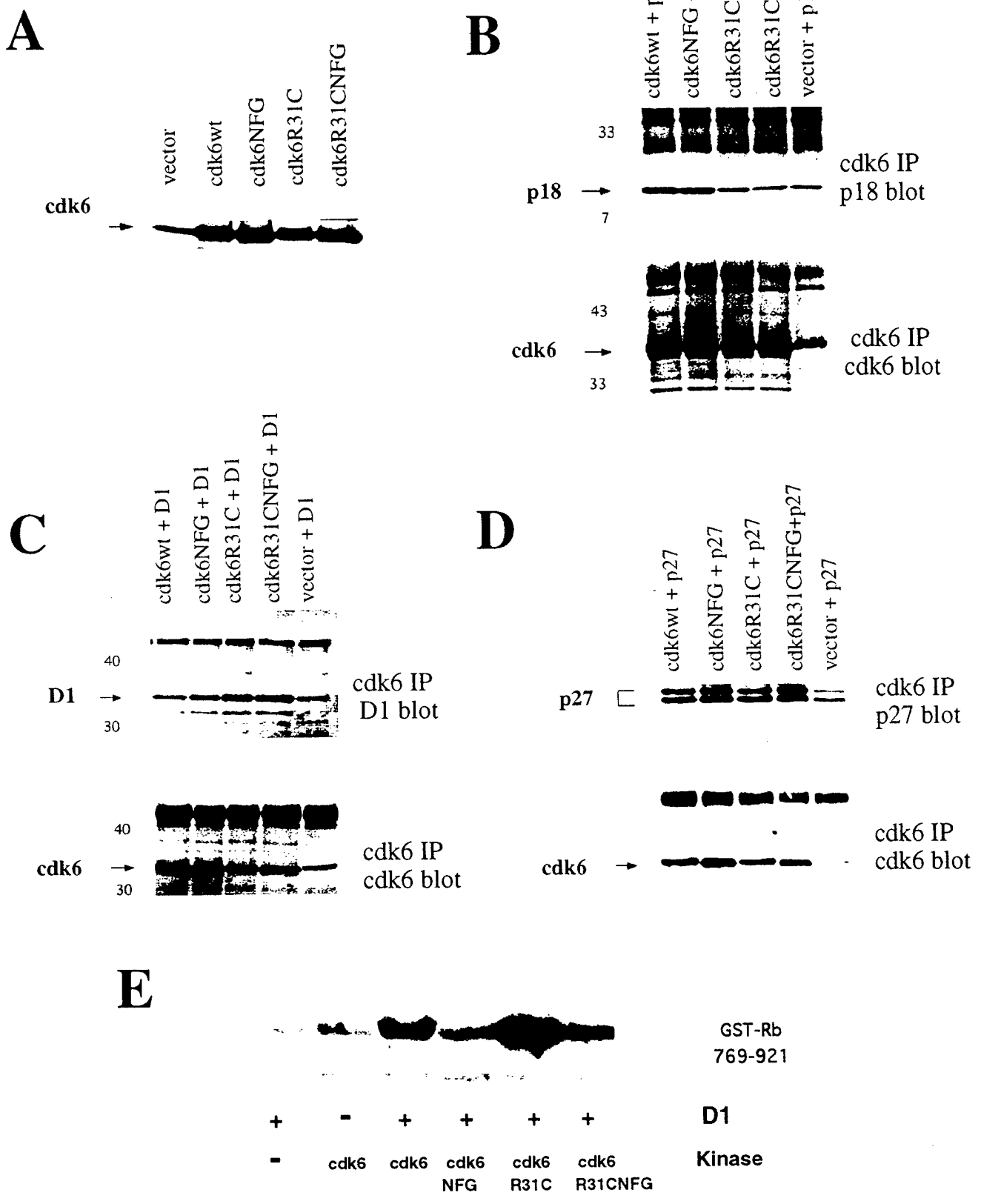
4 hours post Mitotic Shake



8 hours post Mitotic Shake






A
vector
cdk6wt
cdk6NFG
cdk6R31C
cdk6R31CNFG
cdk6
B
cdk6wt + p18
cdk6NFG + p18
cdk6R31C + p18
cdk6R31CNFG+p18
vector + p18
33
p18
7
cdk6 IP
p18 blot
43
cdk6
33
cdk6 IP
cdk6 blot
C
cdk6wt + D1
cdk6NFG + D1
cdk6R31C + D1
cdk6R31CNFG + D1
vector + D1
40
D1
30
cdk6 IP
D1 blot
40
cdk6
30
cdk6 IP
cdk6 blot
D
cdk6wt + p27
cdk6NFG + p27
cdk6R31C + p27
cdk6R31CNFG+p27
vector + p27
p27
cdk6 IP
p27 blot
cdk6
cdk6 IP
cdk6 blot
E
GST-Rb
769-921
+
-
+
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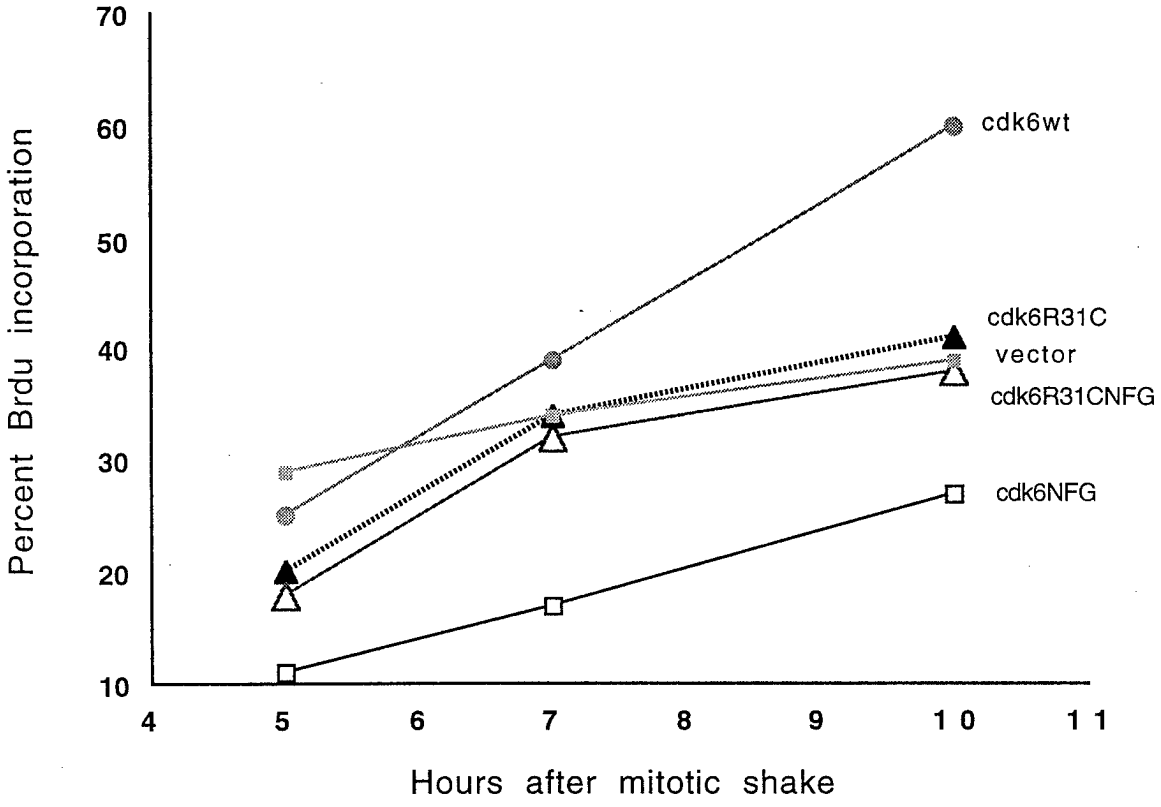
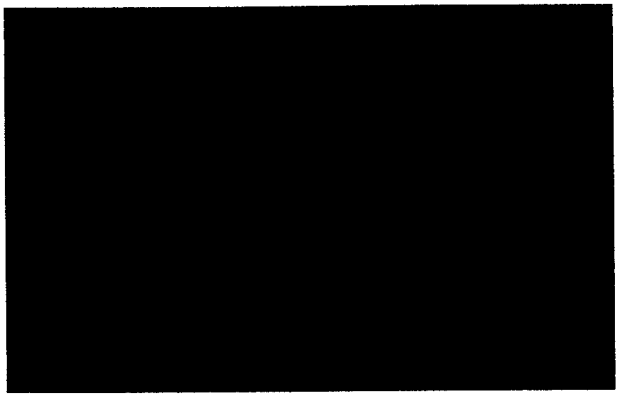
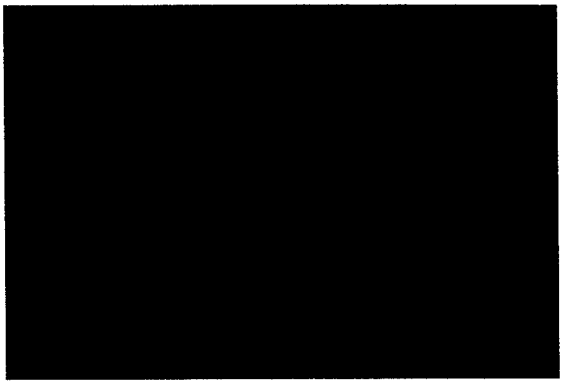
NFG
cdk6
R31C
cdk6
R31CNFG
Kinase


70
60
50
40
30
20
10
Percent Brdu incorporation
4
5
6
7
8
9
10
11
Hours after mitotic shake
cdk6wt
cdk6R31C
vector
cdk6R31CNFG
cdk6NFG

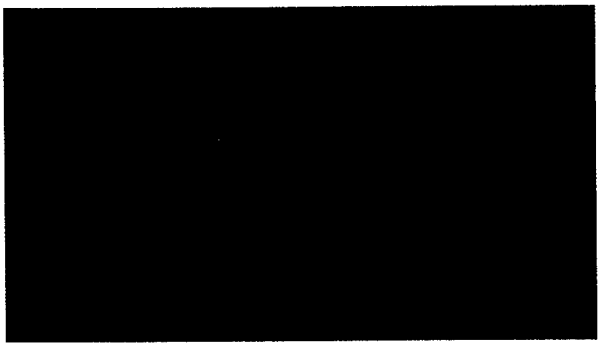
Polyclonal anti-cdk6



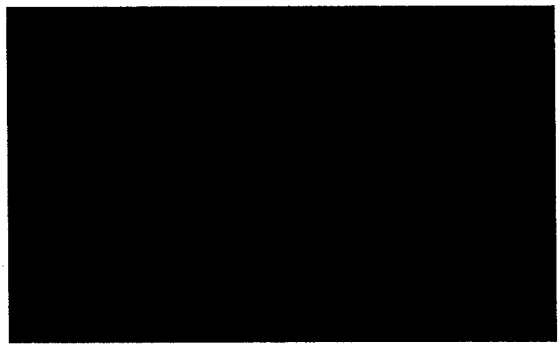
CDK6WT



CDK6NFG

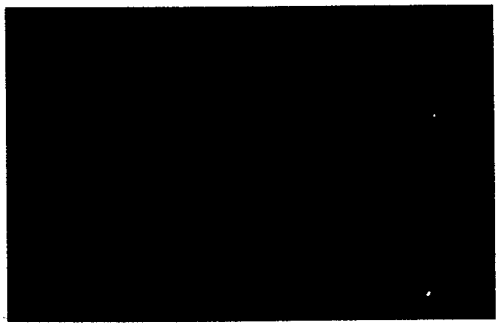


cdk6R31C

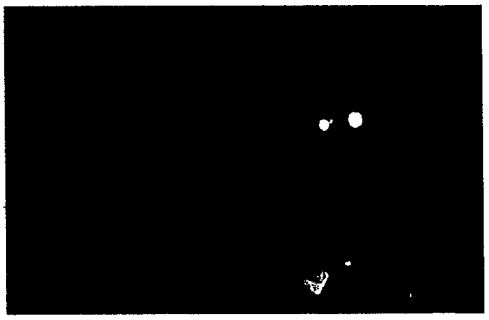


cdk6R31CNFG

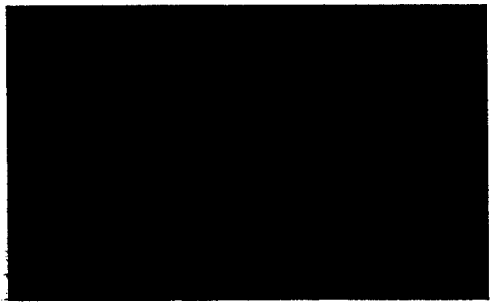
Monoclonal anti-cdk6



Hoechst



cdk6WT



cdk6R31C

A Yeast Two-Hybrid System for Discerning Differential Interactions Using Multiple Baits

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Abstract

The two-hybrid system is a powerful genetic system to identify protein-protein interactions or to study the disruption of protein-protein interactions. To enhance the power of this genetic method we have developed a differential two-hybrid system that allows screening of cDNA libraries with two bait proteins in a single yeast. In this novel system each bait fusion is tethered to a separate DNA-binding domain and activates bait-specific reporter genes, thus allowing the isolation of protein partners that interact with either one or both of two discrete baits after a single library transformation. This system can also be used to identify binding partners specific to either a wildtype or (disease-related) mutant form of a protein or to identify compounds that specifically disrupt interactions with one but not both baits. The differential two-hybrid also allows the identification of a mutant protein that has lost the ability to bind a specific partner while preserving other protein interactions, allowing selection against mutations that cause global conformational disruptions. Finally, this system is well suited for single-step, high-throughput library screens as well as screens of peptides or compounds that disrupt prey interactions with only one of two closely-related baits.

Introduction

The yeast two-hybrid system is a powerful genetic system designed to identify novel protein-protein interactions that were previously limited to detection by biochemical studies. The original system (1) has been manipulated and improved upon by interchanging DNA-binding domains (2), by addition of multiple reporter constructs (2-4), and by using a reverse system to study disruption of protein interactions (4, 5). All two-hybrid screening systems rely on the fact that transcriptional activation and DNA-binding domains of transcription factors are modular in nature (6, 7). In these systems, the coding sequence for the DNA-binding domain of a transcription factor such as Gal4 or LexA is fused to the cDNA of a protein of interest, termed "the bait". The fusion protein thus encoded tethers the bait to the promoter region of a reporter gene. A second fusion of a cDNA library with the coding sequence of a transcriptional activation domain is termed the "prey". Functional reconstitution of transcription factor activity occurs upon association of the bait and prey protein domains. This interaction is then detected by expression of reporter genes that are dependent upon the bait's DNA binding domain.

The two-hybrid system is thus an ideal system to screen libraries for novel protein-protein interactions and is also a powerful tool to isolate factors that disrupt or promote interactions of proteins. The advantage of this system is that an interaction of the bait with an unknown prey leads to direct identification of the cDNA that encodes the interacting protein. This system is made even more powerful by its use in high-throughput screening applications, allowing the screening of large numbers of potential interacting cDNAs or peptides rapidly and efficiently.

Many proteins screened in the two-hybrid system are members of larger protein families. It is often of interest to screen closely related family members for their ability to interact with binding partners identified in the original screen. A system that would permit the screening of two homologous bait proteins in one yeast would allow direct comparisons of binding partners within a family of proteins. This system would be more efficient and would permit the simultaneous identification of proteins that interacted with either one of the two bait proteins and at the same time, proteins that interacted with both of the related baits. This two-hybrid system could also be used to discern similarities and differences in binding partners of a wildtype and mutant form of a protein.

In this report we describe a novel, "differential" two-hybrid yeast system that allows screening for interactions between prey proteins and two different bait proteins through the activation of bait-specific reporters. This system allows the identification of proteins that interact differentially with a bait tethered to the Gal4 DNA-binding domain and a bait tethered to the LexA DNA-binding domain. This system will also allow the identification of factors that specifically disrupt protein-protein interactions of either or both bait proteins.

Methods

Yeast Strains

MaV52 (*MAT***a** *ura3-52 leu2-3,112trp1-901 his3 Δ200 ade2-101 gal4 Δ gal80 Δ* $can1^R$ $cyh2^R$ *GAL1::HIS3@LYS2 GAL1::lacZ*) was a kind gift of Dr. Marc Vidal and is further described elsewhere (4). HW18 (*SPEX8::URA3*) and WY11 (*SPEX4::URA3*) are isogenic derivatives of

MaV52 containing *SPO13*::*URA3* fusions with 8 and 4 LEXA binding sites, respectively.

Construction and integration of SPEX::*URA3 reporter genes*

The plasmid, pMV252 (a kind gift of M. Vidal), used to clone the LEXA sites into the *SPO13*::*URA3* promoter sequence is described elsewhere (4). pMV252 contains *Eco*RI sites at positions -170 and -368 in the *SPO13* promoter. Oligo LAFW: AATTCTGCTGTATATAAAACCAGTGGTTA TATGTACAGTACTGCTGTATATAAAACCAGTGGTTATATGTACAG TACG and oligo LARV: AATTCGTACTGTACATATAACCACTGG TTTTATATACAGCAGTACTGTACATATAACCACTGGTTTTATATA CAGCAG containing *Eco*RI sites were annealed and inserted into the *Eco*RI sites in pMV252. WY11 contains one copy of annealed LAFW and LARV, yielding 4 LEXA binding sites while HW18 contains two concatamers of LAFW and LARV, resulting in 8 LEXA sites. PCR was performed across the *SPO13* promoter and into the *URA3* coding sequence with the 5' oligo JB516: 5' GAAGGTTAATGTGGCTGTGGTTTC AGGGTCCATAAAGCTTGTCCTGGAAGTCTCATGGAG3' and the 3' oligo LEXPCR3: 5'CTAGGTTCCTTTGTTACTTCTTCCG3' (4, 8). The PCR products were transformed into MaV52 containing the Lex-based transcription factor pSH17-4. Integration at *URA3* was selected for by transcription factor-dependent growth on uracil dropout plates and by lack of growth on .2% 5-fluoroortic acid (FOA) plates. PCR from the yeast chromosome confirmed correct integration and allowed determination of the number of LEXA sites inserted. Oligos used were: ura3 5': 5'GCGAGGCATATTTATGGTGAAGG3' and ura3 3': 5' CATTTCCGTGCAAGGTACTAAC3' (4, 8). To lyse yeast for PCR

reactions, a pinhead-sized quantity of yeast was suspended in 5 units/µl of zymolyase solution in water (Seikagaku corporation). The yeast were digested at 37°C for 30 minutes, phenol/chloroform extracted and ethanol precipitated. The DNA pellet was resuspended in 50 µl Tris-HCl (pH8.0) and 10 µl was used in each PCR reaction.

Plasmid Constructions

The plasmid pGalcdk6 contains the BamHI fragment of cdk6 from pCMVcdk6 (9) inserted into the BamHI site of the plasmid pPC97BI. pPC97BI contains a BamHI site inserted into the polylinker of pPC97. pPC97 (4), was digested with SalI and BglII, dropping a small insert. Oligos 97BIFW 5'TCGACCGGATCCCC3' and 97BIRV 5'GATCGGGGATCCGG3' were annealed and ligated to pPC97 to create an additional BamHI site, allowing in-frame fusion of the GalDBD with the cdk6 cDNA. Partial BamHI digestion of pPC97BI allowed the insertion of the BamHI insert into the correct site. The plasmid pLexcdk4 was cloned into pHybLexZeo (Invitrogen, USA). The BamHI fragment containing the cdk4 cDNA from pCMVcdk4 (9) was treated with the DNA polymerase (Klenow fragment) to create blunt ends. pHybLexZeo was digested with SacI and treated with T4 polymerase to generate blunt ends. Ligation of cdk4 and pHybLexZeo blunt ends resulted in the correct reading frame fusion as confirmed by sequence analysis. pLexcdk6 was generated by subcloning the BamHI fragments of cdk6 from pCMVcdk6 into the BamHI site of pSP72. The cdk6 cDNA was removed by KpnI and PvuII digestion and cloned into the KpnI/PvuII sites of pHybLexZeo to create an in-frame fusion of the LexDBD and the cdk6 cDNA. p16trp was generated by EcoRI/XhoI digestion of pCMVp16A (10) and ligated into the EcoRI/XhoI

sites of pJG4-5. Plasmid p18trp was generated from pUCp18 (a kind gift of Y. Xiong). pUCp18 was digested with HindIII and treated with DNA polymerase (Klenow fragment) to the HindIII site and then digested with EcoRI to drop the p18 fragment. The p18cDNA was then inserted into the pJG4-5 that had been digested with EcoRI and XhoI, the XhoI site having been treated with DNA polymerase to create blunt ends. The blunt-ended ligation resulted in an in-frame fusion of p18 with the B42 transactivation domain. Both p16TAD and p18TAD in pJG4-5 are under the control of a galactose-inducible promoter. However, immunoblots of p16 demonstrated that p16 protein was present when yeast were grown either in glucose or in galactose. Thus, the galactose-inducible promoter in pJG4-5 (114bps) is leaky, functioning even in the absence of galactose. When expressed in HW-18 (in which GAL4 and GAL80 are deleted), p16TAD protein levels were enhanced when co-transfected with pGALcdk6, indicating that interaction of p16 (and p18) with Galcdk6 allows increased transcription from the pJG4-5 promoter. Plasmid pYestrp is commercially available (Invitrogen) and plasmid pSH17-4 was a kind gift of Roger Brent and is described elsewhere (6, 11)

Immunoblots and reporter assays

For immunoblots yeast were grown in selective media with galactose to an OD_{600} of approximately .5 and one ml of yeast were pelleted and resuspended in 50µl of 2X SDS/PAGE sample buffer. Yeast were frozen in liquid nitrogen for 20 minutes followed by boiling for 5 mins. 15µl of yeast were loaded per lane onto 10% polyacrylamide gels for immunoblot analysis. Proteins were transferred to supported nitrocellulose (GIBCO BRL) and blotted using antisera as noted. To assay ß-galactosidase activity

(lacZ reporter), filter lift assays were performed. A nitrocellulose filter (MSI, NitroPure) was quick frozen in liquid nitrogen to lyse lifted yeast colonies from the SC-L-T+Zeo GAL/RAF master plate. The activity of ß-galactosidase was tested using 1500µg of the substrate X-GAL, and incubated for one hour to overnight at 30°C.

Results

Differential yeast strain HW18

A yeast strain was constructed in which the transcription of two reporter genes are dependent upon GAL4 binding sites in their promoters and transcription of the third reporter gene is dependent on LEXA binding sites in the promoter region (Figure 1A). This yeast strain allows screening of two different proteins or "baits" fused to two different DNA-binding domains, each bait activating heterologous (GAL4-driven or LEXA-driven) reporter genes. When the GAL4 DNA-binding domain fusion protein (GAL DBD) interacts with a cDNA-library-encoded protein fused to a trans-activation domain (TAD), this protein-protein interaction activates two GAL4-dependent reporter genes: *lacZ* and *HIS3*. A second bait fused to the LexA DNA-binding domain (LEX DBD) activates the LEXA-dependent reporter, *URA3*, when it interacts with a prey-TAD fusion protein. Thus, a two-hybrid screen can be performed with two different baits at the same time with each bait activating individual reporter genes. The strain described here, HW18, contains LEXA binding sites in the *URA3* promoter and GAL4 binding sites in the *HIS3* and *lacZ* promoters (Figure 1A). HW18 is derived from MaV52 (4) by inserting 8 LEXA binding sites into the highly repressive *SPO13* promoter driving the *URA3* open reading frame. DNA gel analysis of PCR reactions that specifically amplified the *URA3* promoter region from the yeast chromosome confirmed that yeast strain WY11 contains 4LEXA binding sites and strain HW18 contains 8 LEXA binding sites (Figure 1B). Importantly, this *SPO13* promoter of *URA3* confers a Ura minus phenotype in the absence of a LexA-based transcription factor.

Plasmids that encoded fusions of cdk6 and cdk4 cDNA's with genes for Gal4 or LexA DNA-binding domains were introduced into the HW18 yeast strain (Figure 1C). The pGALcdk6 plasmid contains the Gal4DBD in reading frame with the cDNA of cdk6 and the *leu2* marker for auxotrophic selection. pLEXcdk4 contains the LexA DBD in frame with the cdk4 cDNA and contains the *zeocin* (*zeo*) resistance selectable marker. In addition, plasmid pLEXcdk6 contains the cdk6 cDNA in frame with the LexA DBD. The third plasmid in these studies contains cDNA's of known cdk4 and cdk6 interacting proteins in frame with B42 TAD and the *trp1* marker. Proteins well-characterized in their ability to bind cdk4 or cdk6 were used to test this novel two-hybrid system. Plasmids encoding the cDNA's of these proteins, p16TAD and p18TAD, were cloned into pJG4-5. Yeast strain HW18 was transformed with these *zeo, leu2* and *trp1* plasmids and selected on SC-L(leu), -T(trp) +zeo (300 ug/ml) media and tested for activation of the three reporter genes.

Activation of GAL4 and LEXA-dependent reporter genes

To examine the specificity of reporter activation, HW18 transformed strains were assayed for the ability to activate the LEXA- and GAL4-dependent reporter genes. Strains used contained the plasmids pGALcdk6, pLEXcdk4 and either p16TAD or the empty vector containing the B42TAD but no cDNA fusion. These strains were tested for Gal4-driven *HIS3* and *lacZ* expression and for LexA-driven *URA3* expression. Figure 2A clearly demonstrates that interaction of cdk6 with p16 activated the *HIS3* gene as evidenced by growth on -HIS + 20mM 3-amino-1,2,4-triazole (3-AT) plates. *HIS3* encodes an enzyme activity that is inactivated by 3-AT, allowing the titration of *HIS3* activity using 3-AT. In addition, the

cdk6-p16 interaction also activated the GAL4-driven *lacZ* gene as evidenced by blue color formation in the yeast. The activation of both GAL4-dependent reporters was dependent on the cdk6-p16 protein-protein interaction since a vector containing the B42TAD but no cDNA (pYEStrp) did not activate the reporter gene. The p16 protein also interacted with the Lexcdk4 fusion protein as evidenced by its growth on the auxotrophic marker plate lacking uracil (-ura) and by its sensitivity to .2% 5-fluoroorotic acid (FOA). Because the product of the *URA3*-encoded enzyme can catalyze the transformation of FOA into a toxic compound, FOA can be used as a negative selection for uracil production (12). Importantly, the empty trp vector containing the TAD but lacking a cDNA did not allow significant growth on plates lacking uracil and did not render the yeast sensitive to FOA. This demonstrates that an interaction between Lexcdk4 and a prey-TAD fusion is required to activate transcription of *URA3*. Immunoblots shown in Figure 2B demonstrate the expected sized protein products of pGALcdk6, pLEXcdk4 and the p16TAD fusions. In order to compare the strength of the cdk4 and cdk6 interactions with p16, cdk6 was also fused in frame to the LexA DBD. Figure 2A demonstrates that the interaction of p16TAD with Lexcdk4 is comparable to the interaction of p16TAD with Lexcdk6 as measured by growth on uracil dropout plates and sensitivity to .2% FOA. Immunoblots shown in Figure 2B demonstrate the expected protein product of Lexcdk6, which migrates more slowly than Galcdk6, indicative of its slightly larger size. The production of uracil in the two strains shown in figure 2A indicates that the interaction of the p16 protein with cdk4 and cdk6 is comparable in this two-hybrid system. Importantly, regardless of the fusion made, a protein-protein interaction is required for uracil production since the DNA-binding

domain itself, even in the presence of the trans-activation domain is unable to activate transcription.

Discriminating interactions of a prey with two different baits

This system was designed to differentiate the interaction of a prey protein with two different bait proteins, thus allowing the identification of a cDNA that encodes a protein that interacts with either one or both of the bait fusions. To test the utility of this system for such applications, the interaction of two different preys was tested for interaction with Galcdk6 and Lexcdk4. As shown in figure 3A, while p16 interacts with both Galcdk6 and Lexcdk4, the p18 protein preferentially interacts with cdk6. This differential interaction is easily revealed by the phenotype of the yeast containing the interactors. Upon interaction with Galcdk6, both p16 and p18 activated the *lacZ* gene and the *HIS3* gene as evidenced by blue color formation and growth of 20mM 3-AT plates. As was previously shown in figure 2A, the activation of both reporters was dependent upon protein-protein interactions as evidenced by a lack of activation in the empty plasmid control strain, which contains the B42 TAD but no cDNA fusion (Figure 3A). The p16 protein also interacted with Lexcdk4 to activate the *URA3* gene as demonstrated by growth on uracil dropout plates and by lack of growth on .2% FOA plates. When the p18 fusion was transformed into yeast containing Galcdk6 and Lexcdk4 the yeast scored positive for lacZ production and grew on 20mM 3-AT media. These Gal4 driven reporters indicate that the cdk6/p18 interaction is clearly able to reconstitute transcription factor activity although the p18 interaction with Galcdk6 may be slightly weaker than the p16 interaction with Galcdk6. Most importantly, p18TAD could not productively interact with Lexcdk4

in the same strain. The p18 transformed strain did not interact with Lexcdk4 as evidenced by lack of growth on ura dropout plates (-ura) and by resistance to .2% FOA. Immunoblots shown in Figure 3B demonstrate that Lexcdk4 and p18 protein are present in this strain, confirming that the p18 protein was produced, yet was unable to interact with Lexcdk4. This finding is consistent with a previously published report indicating that p18 binds cdk6 but not cdk4 in a two-hybrid system (13). These findings indicate that this yeast system is suitable for detecting differential interaction of a bait protein (p18) with two different prey proteins (cdk4, cdk6).

Discussion

A new system for analyzing protein-protein interactions in yeast is described here. This system expands upon the system of Vidal et al. (8) by introducing LEXA binding sites into the promoter region of the *URA3* gene. Thus, the system described here contains two Gal4-dependent reporter genes and one LexA-dependent reporter gene. This combination of differently-activated reporters allows the study of multiple protein-protein interactions in one strain of yeast and in one screening. This system was designed to allow screening of cDNA libraries with multiple baits. When used in "differential" screens, the two Gal4-driven reporters and the single LexA-driven reporter optimize the identification of binding partners for the Gal4 DBD fusion protein and selection for or against the LexA DBD fusion protein. Indeed, this system was designed to allow screening for proteins that interact with cdk6, but that may or may not interact with its homologue, cdk4. Identification of proteins that bind

either cdk4 or cdk6, but not both may lend insight to mechanisms that separate cdk4 and cdk6 function. The ability to screen two proteins at once also decreases the labor involved in screening and allows the identification of proteins that bind to either cdk6, or cdk4, or both proteins. Finally, this system eliminates the need for mating two yeast to discern interactions with additional proteins (14).

The differential two-hybrid yeast has important applications in mutagenesis studies. For instance, the products of a random mutagenesis of a protein can be screened for the persistence of interaction with one binding partner and the simultaneous disruption of a second binding partner. This would allow identification of a mutant protein that specifically disrupts binding of discrete partners while preserving other interactions, thus selecting against mutations that cause global conformational disruptions of the mutagenized protein. For instance, a standard two-hybrid screen was recently used to isolate peptide aptamers that bound cdk2 (15). In yeast mating experiments, cdk2 mutants were screened to determine the ability of the cdk2 mutants to bind either the peptide aptamer or naturally-occuring CIP inhibitors (15). For studies such as these, the powerful technology of the two-hybrid system can now be enhanced by examining multiple protein interactions or disruptions in one yeast strain in one screen.

Finally, this system should provide a powerful tool to study the promotion or disruption of protein-protein interactions using peptide or small molecule libraries. For instance, it may be possible to identify compounds that affect the interaction of a (disease-related) mutant form of a protein with a binding partner, but not a wildtype form of the protein with the same binding partner. Thus, we believe this system is well suited

for discovery of specific interactors with individual members of families of related proteins as well as for high-throughput studies of factors affecting protein-protein associations.

Acknowledgments

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References

1. Fields, S. &Song, O. (1989) *Nature* **340,** 245-246.
2. Gyuris, J., Golemis, E., Chertkov, H.&Brent, R. (1993) *Cell* **75,** 791-803.
3. Durfee, T., Becherer, K., Chen, P., Yeh, S., Yang, Y., Kilburn, A., Lee, W.&Elledge, S. (1993) *Genes & Dev.* **7,** 555-569.
4. Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E.&Boeke, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10315-10320.
5. Vidal, M., Braun, P., Chen, E., Boeke, J. D.&Harlow, E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10321-10326.
6. Brent, R.&Ptashne, M. (1985) *Cell* **43,** 729-736.
7. Ma, J.&Ptashne, M. (1988) *Cell* **55,** 443-446.
8. Vidal, M. 1998. The Reverse Tow-Hybrid and Several of Its Applications.

9. van den Heuvel, S.&Harlow, E. (1993) *Science* **262,** 2050-2054.
10. Medema, R. H. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **14,** 6289-93.
11. Hanes, S.&Brent, R. (1989) *Cell* **57,** 1275-1283.
12. Boeke, J., Lacroute, F.&Fink, G. (1984) *Mol. Gen. Genet.* **197,** 345-346.
13. Guan, K.-L., Jenkins, C., Li, Y., Nichols, M., Wu, X., O'Keefe, C., Matera, G.&Xiong, Y. (1994) *Genes & Dev* **8,** 2939-2952.
14. Finley, R.&Brent, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12980-12984.
15. Cohen, B., Colas, P.&Brent, R. (1998) *Proc. Nat. Acad. Sci., USA* **95,** 14272-14277.

Figure Legends

Figure 1: Differential yeast strain HW18.

A) Eight LEXA binding sites were introduced into the *SPO13* promoter of *URA3* to create the differential strain HW18. The interaction of a bait fusion with a prey fusion reconstitutes the transcription factor resulting in the indicated phenotype. B) Agarose DNA gel of the PCR products of strains WY11 and HW18. Primers in the *SPO13* promoter and *URA3* reading frame specifically amplified the promoter region containing 4 LEXA sites (WY11) or 8 LEXA sites (HW18), resulting in slower migration of the HW18 PCR-amplified DNA product. C) Plasmids pLexcdk4, pLexcdk6, pGalcdk6 and p16TAD or p18TAD were constructed and expressed as described in materials and methods.

Figure 2: GAL4- and LEXA-dependent reporter genes.

A) Yeast strain HW18 was transformed with pGALcdk6 (Galk6), pLEXcdk4 (Lexk4), pLEXcdk6 (Lexk6), p16TAD (p16) or the empty

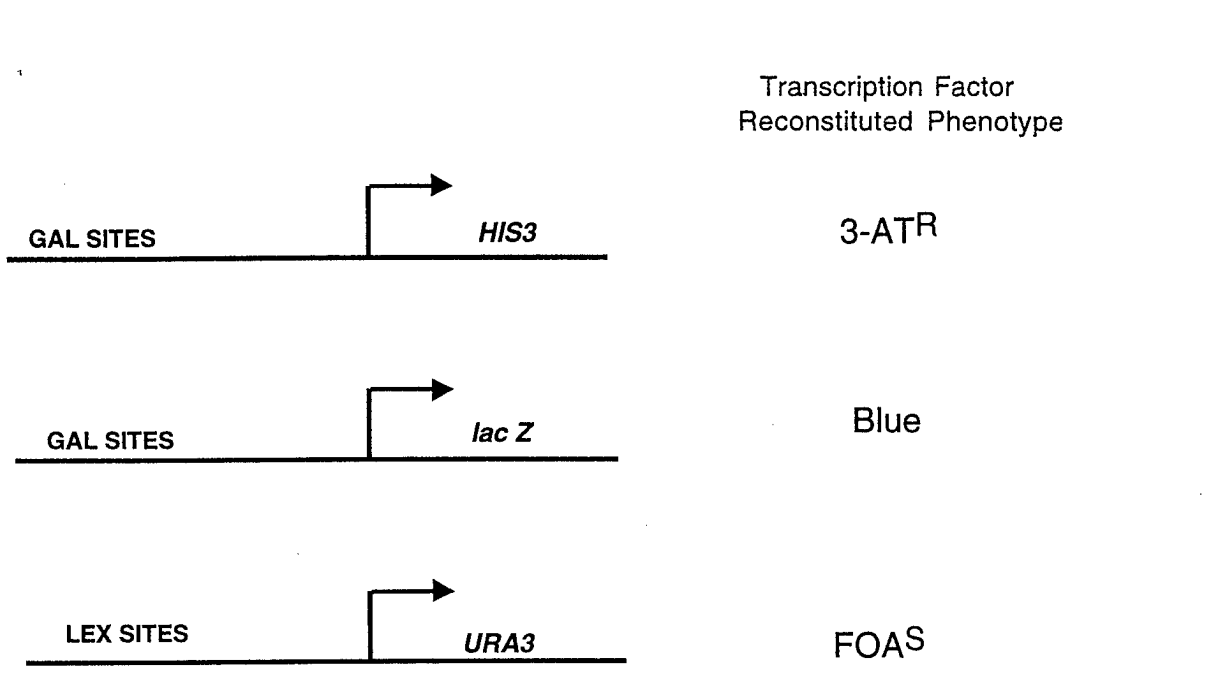
vector pYEStrp (trp). Yeast were streaked onto SC-L-T+Zeo (Glucose) plates for 24 hours and the replica-plated to Gal plates (SC-L-T+Zeo+GAL/RAF) for 24 hours. The Gal plate (Master) was then replica-plated to the indicated reporter dropout plate (Gal) and immediately replica-cleaned to decrease the yeast inoculum. In addition, the -HIS, 3-AT plate was again replica-plated at 24 hours. The Gal Lift reports *lacZ* activity and is described in materials and methods. B) Immunoblot: Yeast strain HW18 (lane1) was transformed with the indicated plasmids and immunoblots were performed as described in materials and methods. Immunoblots are not quantitative, equal volumes (15µl) of yeast extract were loaded in each lane and yeast OD_{600} at time of harvest varied from approxiamtely .6 to .8. Approximate size markers (in kDa) are noted to the left of the panels. The same blot was stripped and reprobed using the indicated antibodies.

Figure 3: Differential interactors.

A) Yeast strain HW18 was transformed with pGALcdk6 (Galk6), pLEXcdk4 (Lexk4), p16TAD (p16), p18TAD (p18) or the empty vector pYEStrp (trp). Yeast were streaked onto SC-L-T+Zeo (Glucose) plates and replica-plated to Gal plates after 24 hours (SC-L-T+Zeo+GAL/RAF). Following incubation for 24 hours, the Gal plate (Master) was then replica-plated to the indicated reporter dropout plate (Galactose) and immediately replica-cleaned. The -HIS, 3-AT plate was also replica-plated at 24 hours. The Gal Lift assay is described in materials and methods. B) Immunoblot of yeast strain HW18 (lane1) containing the indicated plasmids. While equal volumes of yeast extract were loaded, results are not quantitative. OD_{600} of yeast extracts at time of harvest were .6 +/- .1. Approximate

size markers are noted to the left of the panels. A single blot was repeatedly stripped and reprobed using the indicated antibodies.

A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HIS3
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GAL SITES
lac Z
Blue
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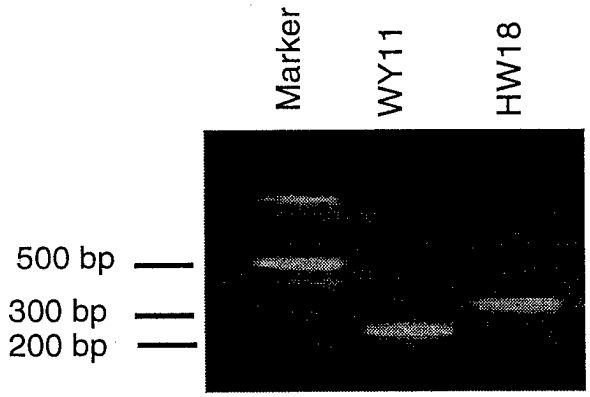
URA3
FOA^S

B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HW18
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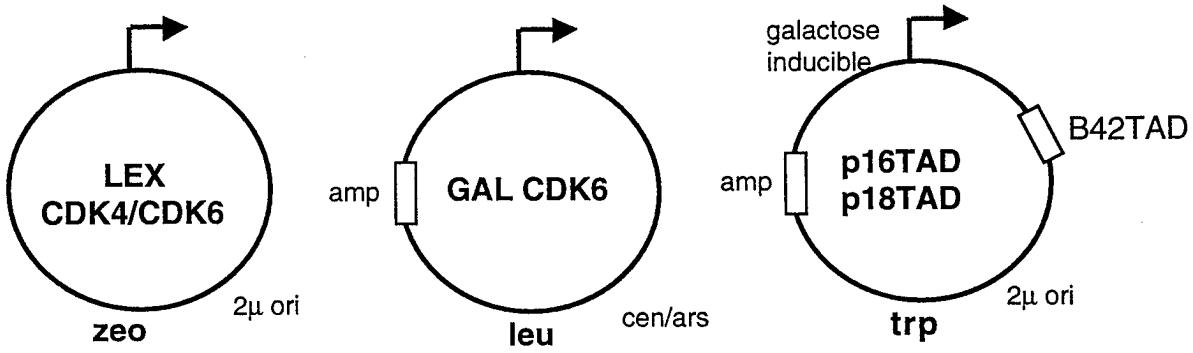
300 bp
200 bp

C


LEX
CDK4/CDK6
2µ ori
zeo
amp
GAL CDK6
leu
cen/ars
galactose
inducible
B42TAD
amp
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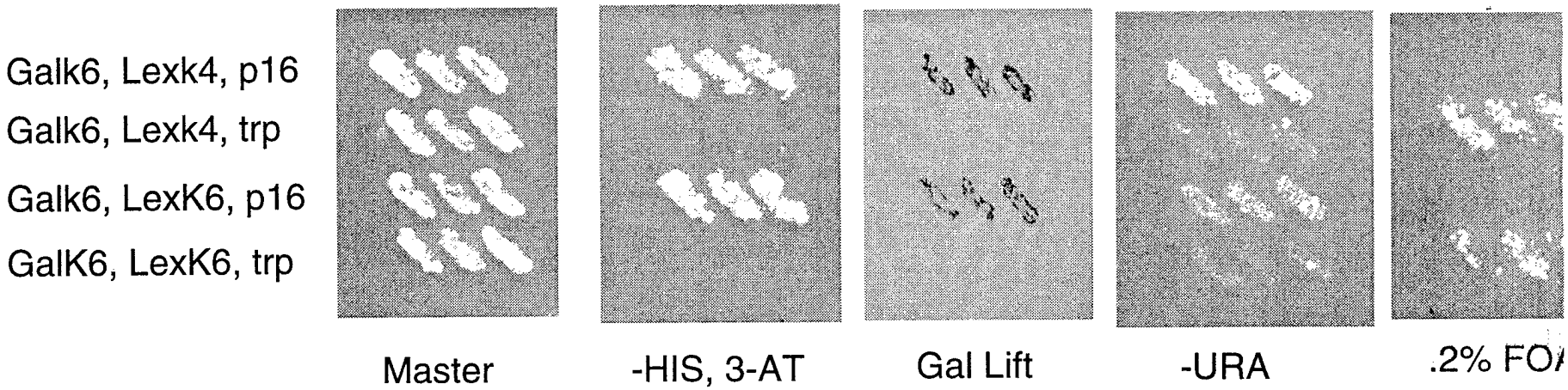
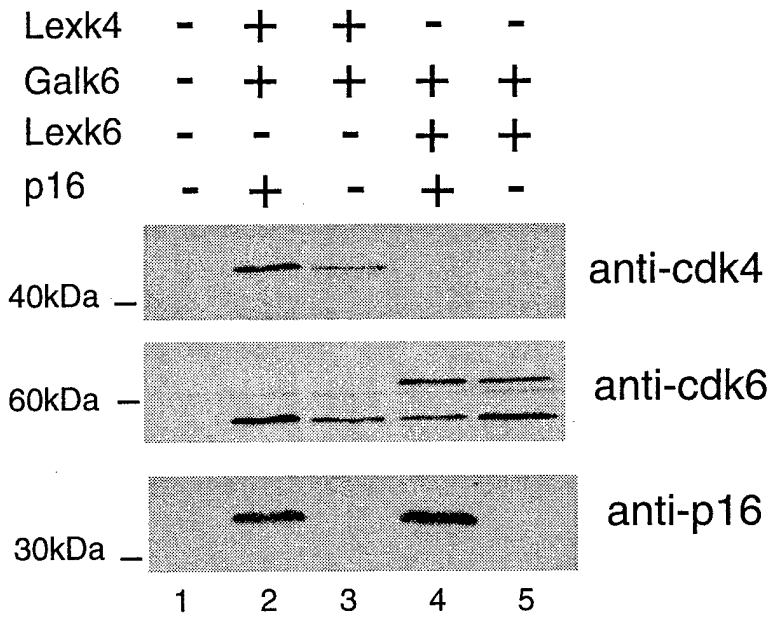
p18TAD
2µ ori
trp

A


Galk6, Lexk4, p16
Galk6, Lexk4, trp
Galk6, LexK6, p16
GalK6, LexK6, trp
Master
-HIS, 3-AT
Gal Lift
-URA
.2% FO

B


Lexk4 - + + - -
Galk6 - + + + +
Lexk6 - - - + +
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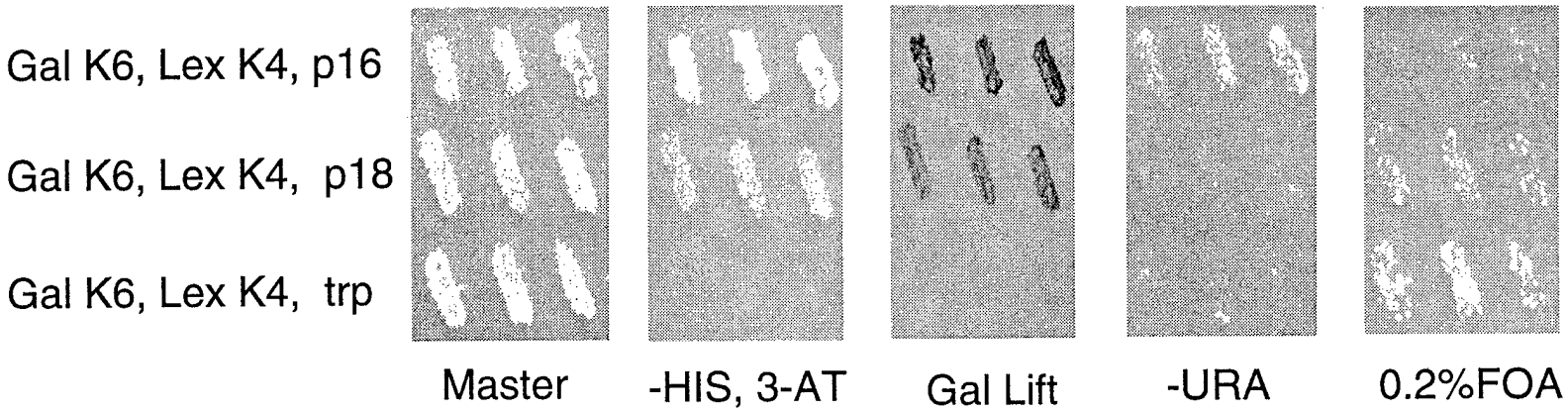
30kDa
anti-p16
1 2 3 4 5

A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Gal K6, Lex K4, p18
Gal K6, Lex K4, trp
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Gal Lift
-URA
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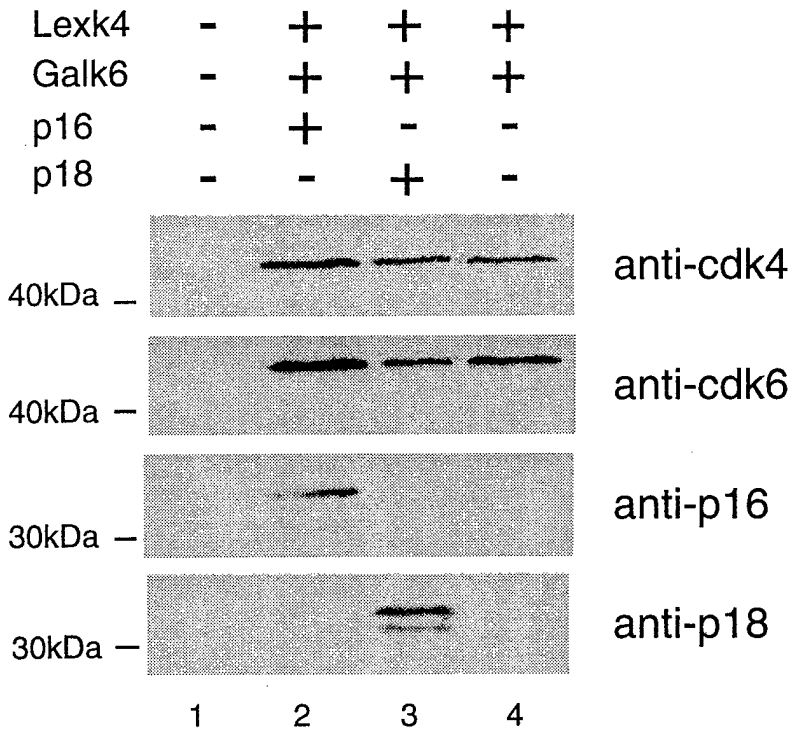
B


Lexk4 - + + +
Galk6 - + + +
p16 - + - -
p18 - - + -
40kDa
40kDa
30kDa
30kDa
anti-cdk4
anti-cdk6
anti-p16
anti-p18
1 2 3 4

Cdk6, but not cdk4 can shorten G1 phase dependent upon the $p18^{INK4C}$ interaction domain

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Abstract:

An increasing body of evidence suggests that certain tumor types specifically activate either cdk4 or cdk6, implying differential function or regulation of these kinases. Cdk6 expression in U2OS cells was found to shorten G1 phase while cdk4 expression did not. The $p18^{INK4c}$ binding domain may play a positive role in cdk6 G1 acceleration since a p18 binding-defective cdk6 mutant (cdk6R31C) was unable to accelerate G1 transit and showed decreased nuclear accumulation. $p18^{INK4c}$ competes with cytoplasmically-localized cdc37 for binding of cdk6, but not cdk6R31C, suggesting a critical role for $p18^{INK4c}$ in generating functional, nuclear cdk6/cyclin D complexes. Cdk6 and cdk4 may respond to different stimuli, playing unique roles in the G1-to-S-phase transition.